UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

Departamento de Sanidad Animal

DESARROLLO DE VACUNAS FRENTE A LA NEOSPOROSIS BOVINA UTILIZANDO AISLADOS DE *NEOSPORA CANINUM* INACTIVADOS Y ATENUADOS

MEMORIA PARA OPTAR AL GRADO DE DOCTOR
PRESENTADA POR

Silvia Rojo Moreno

Bajo la dirección de los doctores

Luis Miguel Ortega Mora
Esther Collantes Fernández

Madrid, 2012

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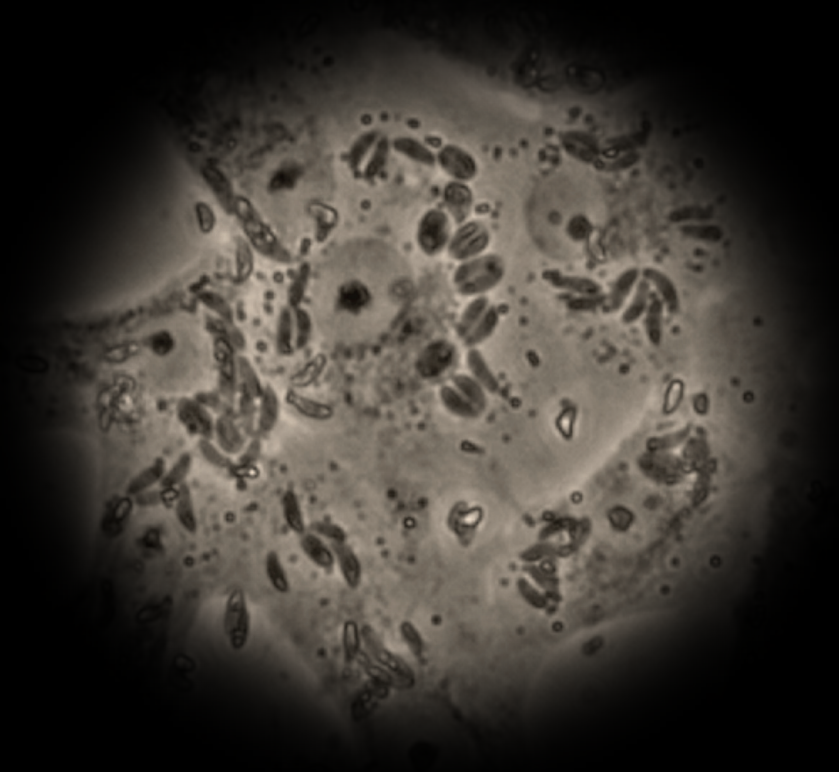

Tesis Doctoral

Silvia Rojo Montejo
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DEVELOPMENT OF VACCINES AGAINST BOVINE NEOSPOROSIS BASED ON INACTIVATED AND ATTENUATED *Neospora caninum* ISOLATES

Doctoral Thesis

Silvia Rojo Montejo

Madrid, 2012

Memoria presentada por Dña. Silvia Rojo Montejo para optar al grado de Doctor por la Universidad Complutense de Madrid

Madrid, diez de enero de 2012

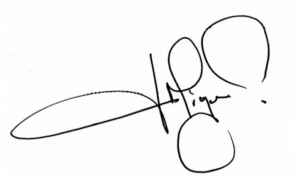
D. Luis Miguel Ortega Mora, Doctor en Veterinaria y Catedrático de Universidad adscrito al Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid y Dña. Esther Collantes Fernández, Doctora en Veterinaria y Profesora Ayudante Doctor adscrita al Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid,

CERTIFICAN:

Que la Tesis Doctoral titulada: **"Desarrollo de vacunas frente a la neosporosis bovina utilizando aislados de *Neospora caninum* inactivados y atenuados"** que presenta la Licenciada en Veterinaria Dña. Silvia Rojo Montejo, para optar al grado de Doctor por la Universidad Complutense de Madrid con Mención Europea, ha sido realizada en las dependencias del Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid bajo su supervisión y cumple todas las condiciones exigidas para optar al grado de Doctor por la Universidad Complutense de Madrid con Mención Europea.

De acuerdo con la normativa vigente, firmamos el presente certificado, autorizando su presentación como directores de la mencionada Tesis Doctoral.

En Madrid, a diez de enero de dos mil doce.



Fdo. Dr. Luis Miguel Ortega Mora

Fdo. Dra. Esther Collantes Fernández

A Marian y Jose

CUANDO BEBAS AGUA, RECUERDA LA FUENTE.

Con el miedo de romper indebidamente el silencio de esta cuartilla, procuraré que estas líneas honren, como es debido, a las muchas personas de las cuales esta etapa es deudora. De todas y cada una he aprendido algo. El ejercicio ahora, es sintetizar esa enseñanza.

Capítulo 1. Mis mentores: *nos esse quasi nanos, gigantium humeris insidentes*.

Luis, son innumerables las lecciones que diariamente nos ofreces a quienes tenemos la fortuna de trabajar contigo. El entusiasmo, la templanza y la voluntad son quizá, las virtudes que más deseo haber interiorizado. Siempre me va a enorgullecer haberme formado al abrigo de un jefe como tú.

Esther, mi guía. La verdad es que no encuentro las palabras que expresen fielmente la gratitud que siento por todo lo que me has regalado a lo largo de estos años. Dudo que vuelva a encontrar una "jefa" que aúne tantas cualidades humanas y profesionales.

Capítulo 2. La familia SALUVET: la Ciencia y la Vida.

Me enfrento a un sentimiento encontrado en este capítulo: la sensación de no haber compartido el tiempo suficiente con las personas que me he ido encontrando en esta casa, y sin embargo he disfrutado y aprendido al máximo de todos y cada uno de los momentos que he vivido con ellos. Agradecérselo es un placer y un deber a partes iguales.

A la Cátedra y demás cabezas pensantes: Mercedes, Isabel, Chema, Ignacio, Cinta y Javier. Son muchas las enseñanzas que he obtenido, como principiante, tras observar el rigor con que un buen investigador trabaja.

A las "hermanas mayores", garantes de profesionalidad, disposición, comprensión y consejo. Susana, Vane, Inmita, Auro y Gema, sois un ejemplo constante. Y ese abrazo que alivia en tiempos de tempestad. Susan, no me puedo olvidar de uno de los acontecimientos más importantes de mi vida: la Antártida. Gracias de corazón por depositar tu confianza en mí.

A Fran-Paca y Sonia-Choni, inmejorables compis de poyata, sufridores de la histeria "pero tú, ¿cuántos guantes utilizas?" y mi tercera mano en este trabajo. Os echo de menos.

A la Ciencia y la Vida: Vero, Adri, Vir, Javi Moreno y su blog, Fran, Claudi, Elena y Gregori. Me alegra mucho haber vivido con vosotros miles de momentos tanto alrededor de la Ciencia como dentro de ella, y al final percibir que todos compartimos el entusiasmo y la voluntad por cambiar las cosas o la capacidad de reírnos de lo absurdo. Sois una de las motivaciones para ir contenta a trabajar.

A SALUVET-Work In Progress: Paula, Alba, Ali, Ofe, Lydia, David, Iván, Dani y a todos los que habéis formado parte en algún momento a lo largo de estos años, en especial a Carmen y Belén. Es una suerte compartir el día a día con gente que hace tan fácil una jornada laboral y cuya manera de ser es un regalo para quien trabaje con ellos.

Al Sr. Lobo, sería injusto no decir nada de la increíble compañía que he tenido durante estos años contigo. No creo que "gracias" sea suficiente.

Capítulo 3. La inestimable colaboración.

A Javier Blanco. Su profesionalidad como veterinario clínico, y su constante apoyo técnico permitió que pudiéramos respirar tranquilos al enfrentarnos a un experimento en un modelo bovino.

A Antonio Rodríguez Bertos, por su labor en el análisis de las lesiones, resolviendo todas mi dudas acerca del insondable mundo de la anatomía patología.

A Ricardo García de la Mata, del Centro de Cálculo de la UCM, por su gran ayuda con el análisis estadístico.

A los veterinarios de la granja Priégola: José Luis Ruiz Castillo, Pablo Rodrigo Martín y José Domínguez Gutiérrez, por su generosa disposición siempre que hemos precisado de su ayuda.

Agradezco al personal de HIPRA su colaboración, y muy especialmente a Toni Prenafeta, por las interesantes y acertadas sugerencias aportadas durante la realización de los experimentos y la corrección de los trabajos.

La realización de esta tesis doctoral ha sido posible gracias al Proyecto PTR1995-0777.OP, subvencionado por HIPRA y el Ministerio de Ciencia y Tecnología, así como a varios proyectos entre HIPRA y la UCM (Artículo 83, LOU 6/2001). Durante estos años, he sido financiada por HIPRA y la UCM (Articulo 83, LOU 6/2001), y a través de un contrato para Personal Investigador de Apoyo de la Consejería de Educación (Comunidad de Madrid).

Chapter 4. The external stay.

I'm deeply in debt to Fiona Tomley, Damer Blake, Virgina Marugán-Hernández, Emily Clark, Sarah Macdonald, Elaine Pegg and all the people from the Royal Veterinary College at University of London. I thank you for your helpful guidance on parasite genetic manipulation technology during my stay in England and for the worthy and joyful time I shared with all of you. Vir, thank you very much to spend a lot of time and effort on this work.

I'm very grateful to Dr. Veronica Risco-Castillo and again to Dr. Fiona Tomley as external expert readers of my doctoral thesis.

Capítulo 5. Antes de los comienzos.

Quiero agradecer con todo mi cariño a aquellos que me mostraron por primera vez el amor por la profesión veterinaria y cuyo consejo fue determinante cuando yo no tenía ni idea de si tomaba la decisión correcta al emprender este apasionante viaje. Gracias a Eutiquio Esteban y Fernando Criado, y de manera muy especial a Juan Vicente González y Susana Astiz, me siento afortunada de teneros como referente.

Capítulo 6. Las raíces.

A mis hermanos de vida, que me habéis aguantado tanto y pedido tan poco. ¿Se puede ser más afortunada? Quiero agradecer especialmente a Anita, Carlos, Anabel, Ana y Yosi. Esto también os pertenece.

Alber y Alex, os agradezco la inagotable paciencia que tenéis y habéis tenido conmigo siempre. No os canséis nunca de tenerla, porfa, sois un motor muy importante de mi vida. Yo a cambio seguiré admirándoos y queriéndoos fielmente como amigos y hermanos mayores. Gracias, también, por regalarme a Rosa y a Bea, y por supuesto, al nuevo cachorro, la pequeña Julia.

Todo lo maravilloso, lo bueno y lo menos bueno que ha rodeado el desarrollo de esta tesis no hubiera sido posible si no hubierais estado incondicional y atemporalmente a mi lado. No creo que haya vidas suficientes para agradeceros, simplemente, todo. Como escribe Saramago: "¿qué otra genealogía puede importarme? ¿en qué mejor árbol me apoyaría?". Gracias, Mamá y Papá.

Índice

Listado de abreviaturas

A_{405}	Absorbancia a 405 nanómetros (*absorbance at 405 nanometers*)
ADN/DNA	Ácido desoxirribonucleico (*deoxyribonucleic acid*)
ADNc/cDNA	ADN complementario (*complementary DNA*)
ADNr/rDNA	ADN ribosómico (*ribosomal DNA*)
Al	Hidróxido de aluminio (*aluminum hydroxide*)
AMA	Antígeno de membrana apical (*apical membrane antigen*)
ANOVA	Análisis de la varianza (*analysis of variance*)
ARN/RNA	Ácido ribonucleico (*ribonucleic acid*)
ARNr/rRNA	ARN ribosómico (*ribosomal RNA*)
ARNm/mRNA	ARN mensajero (*messenger RNA*)
BAG	Antígeno de bradizoíto (*bradyzoite antigen*)
BEI	Etilenimina binaria (*binary ethylenimine*)
BSA	Albúmina sérica bovina (*bovine serum albumin)*
BSR	Recombinante específica de bradizoíto (*bradyzoite specific albumin*)
BZ	Bradizoíto (*bradyzoite)*
CpG-ODN	*Cytosine-phosphate-guanosine oligodeoxynucleotides*
Ct	Ciclo umbral (*cycle threshold*)
dg	Días de gestación (*days of gestation*)
DIGE	Electroforesis diferencial en gel (*diferencial gel electrophoresis)*
DO/OD	Densidad óptica (*optical density*)
EDTA	Ácido etilendiaminotetracético (*ethylenediaminetetraacetic acid*)
ELISA	Ensayo por inmunoabsorción ligado a enzima (*enzyme-linked immunosorbent assay*)
ESA	Proteínas excretadas y secretadas (*excreted-secreted antigen*)
G	Extracto de Ginseng (*ginseng extract*)
GRA	Proteína de gránulos densos (*dense granule protein*)
IFI	Inmunofluorescencia indirecta (*indirect immunofluorescence*)
IFN-γ	Interferón gamma (*interferon gamma*)
Ig	Inmunoglobulina (*immunoglobulin*)
IL	Interleuquina (*interleukin*)
im	Vía intramuscular (*intramuscular route*)
ip	Vía intraperitoneal (*intraperitoneal route*)
IRPC/RIPC	Índice relativo por cien (*relative index per cent*)
ITS-1	Espacio de transcripción interna-1 (*internal transcribed spacer-1*)
iv	Vía intravenosa (*intravenous route*)
kDa	Kilodalton
MAG	Antígeno de la matriz del quiste (*matrix antigen*)
MIC	Proteína de micronemas (*microneme protein)*
MOI	Multiplicidad óptima de infección (*optimized multiplicity of infection*)
MS	Microsatélites (*microsatellites*)

Nc	*Neospora caninum* (prefijo)
NK	*Natural killer cells*
NSA	Antígeno soluble de *N. caninum* (Neospora *soluble antigen*)
PBS	Tampón fosfato salino (*phosphate buffered saline*)
PCR	Reacción en cadena de la polimerasa (*polymerase chain reaction*)
PDI	Isomerasa proteína-disulfuro (*protein disulfure isomerase*)
$PGF_{2\alpha}$	Prostaglandina 2α (*prostaglandin 2α*)
PI	Post-infección (*post-infection*)
PP	Post-parto (*post-partum*)
PV	Vacuola parasitófora (*parasitophorous vacuole*)
RAPD	Amplificación al azar de ADN polimórfico (*randomly amplified polymorphic DNA analysis*)
ROP	Proteína de cuerpo de roptria (*rhoptry bulb protein*)
RT	Retrotranscripción (*retrotranscription*)
Th	T colaboradores (*T helper*)
TLR	*Toll-like receptor*
TGF	Factor de transformación de crecimiento (*transforming growth factor*)
SAG	Antígeno de superficie (*surface antigen*)
sc	Vía subcutánea (*subcutaneos route*)
SD	Desviación estandar (*standar deviation*)
SNC/CNS	Sistema nervioso central (*central nervous system)*
SNP	Nitroprusiato sódico (*sodium nitroprusside*)
SRS	Superfamilia de secuencias relacionadas con la SAG1 (*SAG1 related sequences*)
TH	Transmisión horizontal (*horizontal transmission*)
TNF	Factor de necrosis tumoral (*tumoral necrosis factor*)
TT	Transmisión transplacentaria (*transplacental transmission*)
TTEn	TT endógena (*endogenous TT*)
TTEx	TT exógena (*exogenous TT*)
TZ	Taquizoíto (*tachyzoite*)
UV	Ultravioleta (*ultraviolet)*
vs	versus
W/O	Emulsión de aceite en agua (*water-in-oil emulsion*)

Capítulo I

1. NEOSPOROSIS BOVINA

La neosporosis bovina es una enfermedad de distribución cosmopolita que ha emergido en las dos últimas décadas como causa importante de fallo reproductivo en las principales zonas productoras de ganado bovino del mundo, originando abortos y nacimiento de terneros con signos clínicos neuro-musculares o clínicamente sanos, pero crónicamente infectados (Dubey, 1999b; Dubey, 2003; Dubey et al., 2007).

El agente causal de la neosporosis bovina es *Neospora caninum,* un protozoo intracelular obligado perteneciente al phylum Apicomplexa. Este parásito fue detectado por primera vez en el año 1984 en Noruega, en perros que presentaban trastornos nerviosos caracterizados por meningoencefalomielitis y miositis (Bjerkås et al., 1984). Posteriormente, varios autores describieron la presencia de un parásito formador de quistes, no identificado hasta el momento aunque similar a *Toxoplasma gondii*, asociado a encefalomielitis en terneros neonatos (O'Toole & Jeffrey, 1987; Parish et al., 1987). Pero no fue hasta 1988 cuando Dubey et al. identificaron y aislaron este parásito en perros que presentaban signos clínicos neuromusculares similares a los observados en 1984 por Bjerkås et al., proponiendo un nuevo género, *Neospora*, con una única especie representativa hasta ese momento: *N. caninum* (Dubey et al., 1988a; Dubey et al., 1988b). En 1989 se describió por primera vez a *N. caninum* como agente causal del aborto bovino (Thilsted & Dubey, 1989). Posteriormente, Anderson et al. (1991) y Barr et al. (1991a) reconocieron a la neosporosis como la principal causa de aborto en el ganado bovino lechero de California, hallazgo que fue confirmado, en otros países, por diferentes grupos investigadores. Desde entonces, el interés en el campo veterinario por este protozoo ha ido en aumento, habiéndose realizado numerosas investigaciones sobre la biología del parásito y sus mecanismos de patogenicidad, así como sobre el diagnóstico, la epidemiología y el control de la enfermedad.

1.1. Agente etiológico

1.1.1. Taxonomía

Neospora es un género conformado por protozoos parásitos que se incluye actualmente dentro del phylum Apicomplexa, clase Sporozoea, subclase Coccidia, orden Eucoccidia, suborden Eimeriina, familia Sarcocystidae, junto con los géneros *Toxoplasma*, *Hammondia, Besnoitia, Sarcocystis* y *Frenkelia* (Dubey et al., 1988a; Ellis et al., 1994). Los integrantes de la familia Sarcocystidae se caracterizan por tener un ciclo biológico heteroxeno y su capacidad de formar quistes tisulares en el hospedador intermediario.

En el género *Neospora* se han descrito hasta la fecha dos únicas especies: *N. caninum* y *Neospora hughesi*. La situación taxonómica de *N. caninum* ha sido cuestionada desde su descripción, siendo objeto de importantes controversias por su estrecha relación filogenética con tres especies de la misma familia: *T. gondii*, *Hammondia hammondi* y *Hammondia heydorni* (Holmdahl et al., 1994; Mehlhorn & Heydorn, 2000; Heydorn &

Mehlhorn, 2002a; Heydorn & Mehlhorn, 2002b). Sin embargo, las importantes diferencias genéticas, biológicas y estructurales encontradas justifican la existencia de esta especie (Guo & Johnson, 1995; Holmdahl & Mattsson, 1996; Ellis et al., 1999; Mugridge et al., 1999; Speer et al., 1999; Mugridge et al., 2000; Dubey et al., 2002).

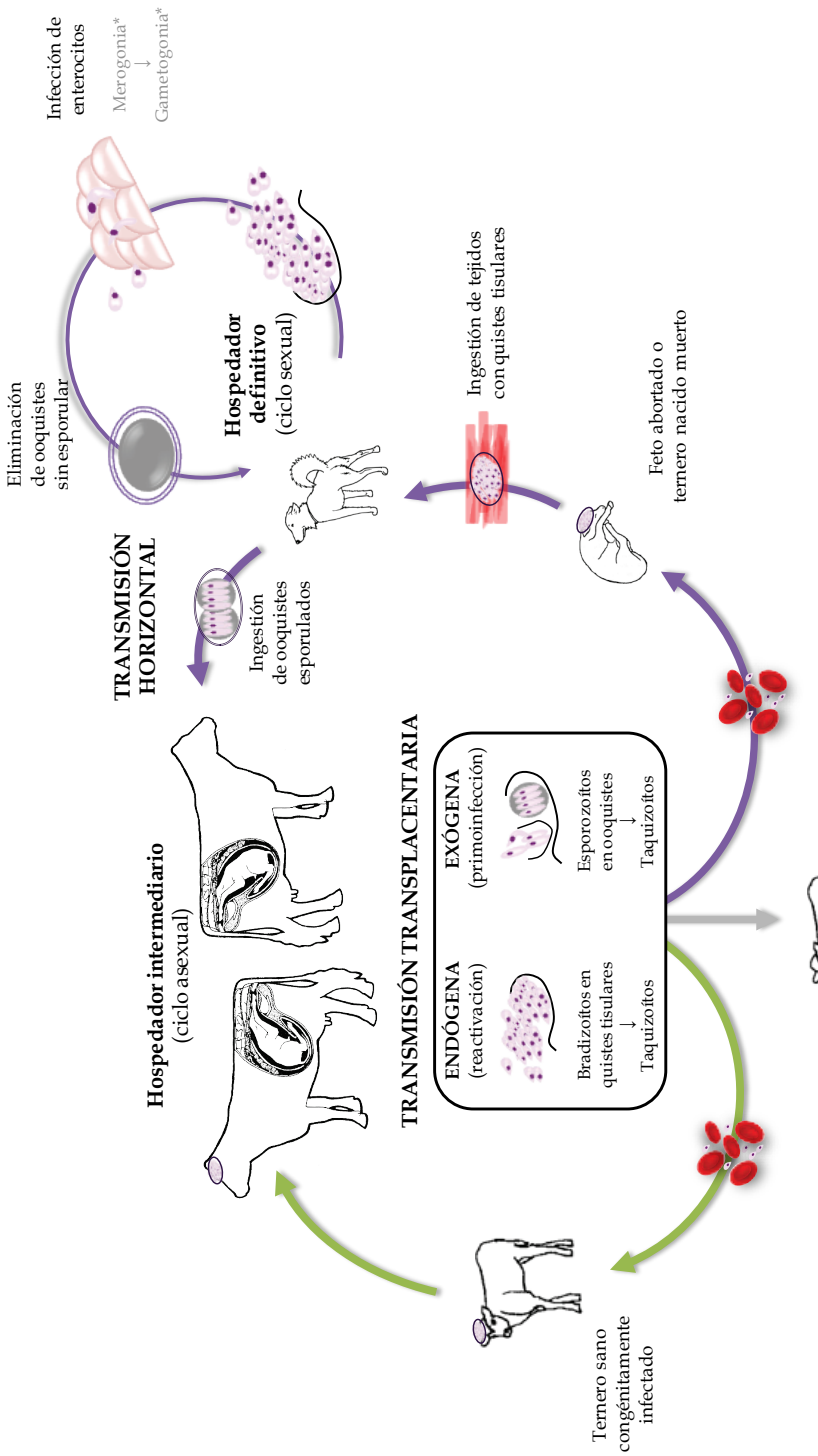
N. hughesi, aislada a partir de tejido nervioso de un caballo adulto que presentaba signos clínicos nerviosos, fue descrita por Marsh et al. (1998). A pesar del escaso conocimiento que se tiene hasta la fecha sobre esta especie, diversos autores han puesto en evidencia las diferencias ultraestructurales, antigénicas, genéticas y patogénicas existentes entre ella y *N. caninum* (Marsh et al., 1998; Walsh et al., 2000).

1.1.2. Espectro de hospedadores y ciclo biológico

El ciclo biológico de *N. caninum* es heteroxeno facultativo, interviniendo dos tipos de hospedadores en los que completa las fases sexual y asexual de su desarrollo (Figura 1). En la actualidad, los únicos hospedadores definitivos conocidos de *N. caninum* son el perro (McAllister et al., 1998), el coyote (Gondim et al., 2004), el dingo (King et al., 2010) y el lobo (Dubey et al., 2011). En lo referente a los hospedadores intermediarios, además de la especie bovina, la infección por *N. caninum* ha sido detectada en una gran variedad tanto de especies domésticas (ej. ganado ovino y caprino, caballo, gato, perro, cerdo, dromedario, llama, alpaca), como silvestres (ej. zorro rojo, lobo, lince ibérico, ciervo común, corzo). No se puede descartar el papel de algunas de estas especies silvestres (zorro rojo o lince ibérico) u otros carnívoros como potenciales hospedadores definitivos, ni el de otras especies domésticas y silvestres como hospedadores intermediarios (Dubey et al., 2007).

En el ciclo biológico de *N. caninum* se han identificado tres estadios diferentes: el esporozoíto, el taquizoíto y el bradizoíto. El esporozoíto es el estadio infectante para los hospedadores intermediarios y está localizado en el interior de los ooquistes esporulados. Los ooquistes se formarían tras la multiplicación sexual del parásito y representan la fase de resistencia de éste en el ambiente. Tienen forma esférica o subesférica, pared lisa, un tamaño de 11,7 µm de longitud y 11,3 µm de anchura y contienen dos esporoquistes elipsoidales con 4 esporozoítos cada uno (McAllister et al., 1998). Por su parte, los taquizoítos y los bradizoítos están presentes en los tejidos del hospedador intermediario. Ambos son estadios asexuales del parásito, su localización es intracelular y conservan la morfología y características ultraestructurales típicas de las fases infectivas de los parásitos apicomplejos. Presentan una forma ovoide, de media luna o globular y las organelas y cuerpos de inclusión propios de estos parásitos: apicoplasto, micronemas, roptrias, gránulos densos y un citoesqueleto altamente especializado compuesto en este caso por microtúbulos subpeliculares, anillos polares anteriores y posteriores, conoide y una película integrada por el plasmalema y el complejo interno de membrana (Dubey & Lindsay, 1996). Los taquizoítos (3-7 × 1-5 µm) representan la fase asexual de multiplicación rápida del parásito que sucede durante la fase aguda de la infección. Estos invaden un amplio espectro de células del hospedador incluyendo células neuronales, endoteliales, miocitos, hepatocitos, células renales, macrófagos o células trofoblásticas (Bjerkås & Presthus,

1989; Speer & Dubey, 1989; Barr et al., 1991b; Dubey & Lindsay, 1993; Dubey et al., 2002). Por su parte, los bradizoítos (6-8 × 1-1,8 μm) son las formas asexuales de replicación lenta y representan el estadio de persistencia del parásito en el hospedador. Los bradizoítos están contenidos en los quistes tisulares que se localizan principalmente en el tejido nervioso del hospedador intermediario (Dubey et al., 1988b; Barr et al., 1992; Kobayashi et al., 2001), y con menos frecuencia en tejido muscular esquelético (Peters et al., 2001; Dubey et al., 2004). Los quistes tisulares son redondos u ovalados, y su tamaño puede variar desde 50 μm hasta 107 μm. La pared quística, con un grosor que oscila entre 2,5 a 4 μm, está constituida por una capa externa primaria proveniente de la vacuola parasitófora y otra capa interna gruesa de estructura vesículo-granular (Barr et al., 1991b; Dubey et al., 2006).



* Por determinar

Figura 1. Ciclo biológico y principales modos de transmisión de *N. caninum*.

Ciclo sexual

El ciclo sexual tiene lugar en el hospedador definitivo (el perro, el coyote, el dingo y el lobo). Este puede adquirir la infección tras la ingestión de tejidos que contengan quistes tisulares, eliminando en sus heces los ooquistes, que contaminan el alimento y el agua de bebida (McAllister et al., 1998; Lindsay et al., 1999a; Lindsay et al., 2001). Después de 24 horas en el ambiente, estos ooquistes inician su proceso de esporulación (McAllister et al., 1998), siendo infectantes por vía oral para el ganado bovino y el resto de hospedadores intermediarios (De Marez et al., 1999). Aunque aún no ha sido estudiado, se postula que los ooquistes, resistentes a los jugos gástricos, pasan al intestino de estos animales donde se desenquistarían liberándose los esporozoítos, que atravesarían la pared intestinal, accediendo a la circulación sanguínea o linfática, donde rápidamente se transformarían a taquizoítos.

Ciclo asexual

Los taquizoítos, responsables de la fase aguda de la infección se diseminan por diferentes tejidos por vía linfática o sanguínea, invadiendo diversos tipos celulares (Dubey et al., 1988b; Bjerkås & Presthus, 1989; Speer & Dubey, 1989; Hemphill, 1999). Los taquizoítos penetran en la célula diana mediante invasión activa, quedando englobados en una vacuola parasitófora, formada conjuntamente por la célula hospedadora y el parásito, en la que el taquizoíto inicia su multiplicación por endodiogenia (Speer & Dubey, 1989), pudiendo albergar una célula más de 100 taquizoítos. Aunque se trata de un parásito intracelular obligado, también puede encontrarse libre tras la ruptura de la célula hospedadora en momentos previos a la invasión de una nueva célula (Hemphill, 1999; Buxton et al., 2002). Esta fase aguda de la infección finaliza cuando los taquizoítos se transforman en bradizoítos, de escasa o nula multiplicación en el interior de quistes tisulares, probablemente como mecanismo de evasión de la respuesta inmunitaria generada por el hospedador, estableciéndose la fase crónica de la infección (Buxton et al., 1998; Buxton et al., 2002). Si durante la gestación, tiene lugar en el hospedador intermediario una primoinfección tras la ingestión de ooquistes esporulados o la reactivación de una infección crónica con diferenciación de los bradizoítos a taquizoítos, éstos pueden alcanzar la placenta por vía sanguínea e infectar al feto en desarrollo (Williams et al., 2009).

1.2. Prevalencia

En la actualidad, son relativamente numerosos los estudios de seroprevalencia de la infección por *N. caninum* en el ganado adulto, obteniéndose tasas de prevalencia de rebaño e individual muy variadas y dependientes de la localización geográfica y de la aptitud del ganado (Dubey et al., 2007). Además, las diferencias entre las técnicas diagnósticas, el diseño de los estudios y el tamaño de muestra empleados, dificultan la comparación entre trabajos y datos obtenidos. En este sentido, en un estudio llevado a cabo en cuatro países europeos (Alemania, Holanda, Suiza y España), se compararon seroprevalencias de granjas tanto de aptitud lechera como cárnica, procedentes de regiones representativas de la producción bovina de cada país, empleando técnicas serológicas estandarizadas y diseños experimentales similares (Bartels et al., 2006a). Los resultados de este

estudio mostraron importantes diferencias en las prevalencias según el país. La seroprevalencia individual fue muy baja en Suecia y Alemania (0,5 y 1,6% en vacas de aptitud láctea, respectivamente) mientras que en Holanda y España fue de moderada a alta (9,9 y 16,2%, respectivamente).

Los datos más recientes de estudios realizados en la cabaña bovina española también muestran altas seroprevalencias. En un estudio realizado sobre ganado bovino de aptitud láctea, se detectaron seroprevalencias de rebaño del 79,3% e individual del 15,7% (González-Warleta et al., 2008). Otro estudio realizado en 2004 sobre explotaciones incluidas en las Agrupaciones de Defensa Sanitaria Ganadera gallegas, mostró una seroprevalencia de rebaño del 87,7% en las de aptitud láctea, 76,7% en las de carne y 78,4% en las mixtas y una seroprevalencia individual del 21,9% en los animales de aptitud láctea, 25,1% en los de carne y 28,6% en aptitud mixta (Eiras et al., 2011).

La participación de *N. caninum* como agente etiológico del aborto en el ganado bovino ha sido descrita tanto en ganado lechero como de carne a nivel mundial (Dubey et al., 2007; Dubey & Schares, 2011). En España, los estudios realizados señalan altas tasas de prevalencia de la infección en fetos abortados, entre 22% y 58%, en dependencia de la técnica diagnóstica utilizada (Aduriz et al., 1999; González et al., 1999; Aduriz et al., 2000; Pereira-Bueno et al., 2003), poniendo de manifiesto la gran repercusión de esta parasitosis.

1.3. Transmisión

El ganado bovino puede adquirir la infección por *N. caninum* principalmente de dos modos: congénitamente (transmisión transplacentaria, TT), cuando en una hembra gestante los taquizoítos atraviesan la placenta e invaden el feto, o postnatalmente (transmisión horizontal, TH), por ingestión de alimento o agua contaminados con ooquistes esporulados (Figura 1). La TT es la vía de transmisión más eficiente del parásito ya que se han detectado porcentajes de transmisión que oscilan entre el 40% y el 95% (Dubey et al., 2007), jugando un papel relevante en la propagación y mantenimiento de la enfermedad en un rebaño. Además, una hembra congénitamente infectada puede transmitir la infección a su progenie repetidamente en diferentes gestaciones, consecutivas o intermitentes, si bien la tasa de transmisión va disminuyendo posteriormente debido al desarrollo de inmunidad (Anderson et al., 1995).

Según su origen, la TT puede ser endógena (TTEn) o exógena (TTEx) (Trees & Williams, 2005). La TTEn ocurre tras la recrudescencia de una infección crónica durante la gestación en una hembra persistentemente infectada. La TTEn está asociada a un patrón endémico de abortos, con una baja tasa de aborto que persiste a lo largo de meses y años (Schares et al., 2002). En cambio, la TTEx ocurre en vacas que se infectan por primera vez mediante TH durante la gestación transmitiendo la infección a su progenie. La TTEx se ha relacionado con abortos de patrón epidémico, con tasas de aborto superiores al 10-12,5% en menos de 6-8 semanas (Dijkstra et al., 2001; Schares et al., 2002). La TTEn aparece como el modo de transmisión predominante en muchos rebaños, mientras que existe controversia en cuanto a la eficiencia de la TTEx para dar lugar a una infección

crónica y desencadenar posteriormente la TTEn en gestaciones futuras (McCann et al., 2007; Dijkstra et al., 2008). Hasta la fecha, sólo se ha logrado reproducir a nivel experimental la TTEx en vacas gestantes tras la infección con taquizoítos u ooquistes, y no la TTEn (Williams et al., 2000; Innes et al., 2001; McCann et al., 2007), lo que permite sugerir un posible fallo en el establecimiento de una infección persistente cuando el ganado se infecta postnatalmente. Sin embargo, en infecciones naturales se ha observado la TTEn en animales infectados postnatalmente (Dijkstra et al., 2008), indicando la persistencia de la infección, aunque como señala el estudio, las tasas de transmisión fueron más bajas (58%) en comparación con las obtenidas en otros estudios en que los animales estaban congénitamente infectados (entre el 81 y el 95%) (Pare et al., 1996; Pare et al., 1997; Schares et al., 1998; Davison et al., 1999).

A pesar de la eficiencia de la TT, se ha señalado mediante modelos matemáticos de simulación que esta vía no es suficiente para mantener la infección en los rebaños bovinos sin la existencia de la TH (French et al., 1999). Recientemente, se ha realizado un estudio sobre 108 rebaños holandeses de aptitud lechera donde se estimó una tasa de incidencia de TH de 1,4 infecciones por cada 100 vacas (Bartels et al., 2007), lo que junto con lo expuesto anteriormente pone de manifiesto la relevancia de la TH como vía de infección en el ganado bovino. En España, el análisis seroepidemiológico de varias granjas de vacuno de leche ha puesto en evidencia la importancia de la TH y la existencia de distintas situaciones epidemiológicas: granjas infectadas con patrón endémico de aborto y predominancia de la TTEn, granjas con patrón epidémico de aborto y predominancia de la TTEx y granjas infectadas de forma crónica pero experimentando simultáneamente la TTEn y la TTEx (Rojo-Montejo et al., 2009).

Otras formas de transmisión como la venérea y la galactófora se han postulado como posibles modos de transmisión, sin embargo, han demostrado ser epidemiológicamente poco importantes (Uggla et al., 1998; Davison et al., 2001; Serrano et al., 2006; Serrano-Martínez et al., 2007). Existen, además, otras potenciales fuentes de infección relacionadas con cánidos silvestres y hospedadores intermediarios silvestres tales como roedores que podrían actuar como reservorio, y cuya interacción con el ciclo doméstico, debe tenerse en cuenta, aunque su importancia epidemiológica está aún por determinar (Gondim, 2006).

1.4. Inmunidad

Los numerosos estudios realizados hasta la fecha han identificado componentes de la respuesta inmunitaria innata y adquirida involucrados en el control de la neosporosis. Los mecanismos para el desarrollo de una respuesta inmunitaria protectora frente a la infección por *N. caninum* se ponen en funcionamiento en los primeros momentos tras la infección. Se ha descrito la activación de diferentes componentes de la inmunidad innata como células dendríticas, células Natural Killer y macrófagos, las cuales responden liberando citoquinas del tipo IL-12 e IFN-γ (Boysen et al., 2006; Strohbusch et al., 2009b; Feng et al., 2010; Teixeira et al., 2010; Dion et al., 2011). El reconocimiento del parásito por parte de las células presentadoras de antígeno y la producción temprana de estas cito-

quinas puede ser importante en el control de la proliferación de los taquizoítos en las primeras fases de la infección. Además, cabe destacar la importancia de esta primera respuesta innata e inespecífica como paso previo a la activación de linfocitos y al consiguiente desarrollo de una respuesta inmunitaria específica con memoria inmunológica frente a *N. caninum*.

La localización intracelular de *N. caninum* sugiere que una respuesta mediada por células es el componente más importante para la protección (Hemphill, 1999; Innes et al., 2000). En este sentido, el control de la infección por este protozoo en el hospedador se ha asociado a una respuesta inmunitaria adquirida de orientación Th1, donde están implicadas diferentes citoquinas y poblaciones de células T (Khan et al., 1997; Hemphill et al., 2006). La importancia del IFN-γ se ha puesto de manifiesto en varios estudios *in vitro* e *in vivo,* donde se ha demostrado la inhibición de la multiplicación de *N. caninum* en cultivo celular por adición de IFN-γ al medio (Innes et al., 1995; Yamane et al., 2000), así como una mayor susceptibilidad a la infección en un modelo murino tras el tratamiento con anticuerpos anti-IFN-γ (Khan et al., 1997; Baszler et al., 1999). A nivel celular, en las primeras fases de la infección, las células T CD4+ son activadas en presencia de IFN-γ, orientando la respuesta inmunitaria hacia un tipo Th1, activando a su vez los linfocitos T CD8+. Estos últimos parecen desempeñar un papel importante durante la fase aguda, ya que actúan como células citotóxicas, limitando la diseminación del parásito (Ritter et al., 2002; Spencer et al., 2005). Sin embargo, en fases más avanzadas, los linfocitos T CD4+ parecen ser más importantes en la protección frente a *N. caninum*, ya que la depleción de estos linfocitos en ratones tratados con anticuerpos monoclonales anti-CD4+ condujo a una elevada morbilidad y mortalidad, mientras que en ratones tratados con anticuerpo anti-CD8+, sólo se observó una mortalidad del 30% (Tanaka et al., 2000; Nishikawa et al., 2001a). Aunque, el papel de estas células en la inmunidad frente a *N. caninum* en el ganado bovino está poco estudiado, se ha demostrado la participación de los linfocitos CD4+ y CD8+ en la producción de IFN-γ en los estadios iniciales de la infección, mientras que sólo los CD4+ predominan en las fases más avanzadas (Marks et al., 1998; Tuo et al., 2005; Klevar et al., 2007). Las células T CD4+ pueden mediar la lisis directa de células infectadas (Staska et al., 2003).

Por el contrario, la sensibilidad a la infección en el modelo murino parece estar relacionada con una respuesta de tipo Th2 y niveles elevados de IL-4. Los ratones C57BL/10ScCr deficientes en el receptor TLR4 y en el receptor funcional de la IL-12, sucumbieron a la infección por *N. caninum* a dosis bajas, demostrándose bajos niveles de IFN-γ y altos de IL-4, a diferencia de los ratones C57BL/10ScSn inmunocopetentes (Botelho et al., 2007). La resistencia natural de la estirpe B10.D2 frente a la infección por *N. caninum* se debe a la falta de producción de IL-4 en el bazo, mientras que los ratones de la estirpe BALB/c mostraron niveles más elevados de esta citoquina, asociados a un aumento en la gravedad de la encefalitis y en la carga parasitaria (Long et al., 1998; Baszler et al., 1999).

La inducción simultánea de IL-10 e IFN-γ es común en la respuesta inmunitaria frente a parásitos intracelulares dado que la producción de IL-10 es necesaria para prevenir los posibles efectos nocivos de una sobreproducción de citoquinas de tipo Th1, au-

mentando así la supervivencia del parásito y la del propio hospedador (Kasper & Khan, 1998; Eperon et al., 1999; Nishikawa et al., 2001a; Quinn et al., 2004).

Por último, el papel de la respuesta inmunitaria humoral en el control de la infección por *N. caninum* es muy discutido, y esta aún por dilucidar. Es posible que los anticuerpos generados específicamente frente al parásito (Long et al., 1998; Baszler et al., 1999; Collantes-Fernández et al., 2006b) tengan varias funciones, tal y como ocurre en la toxoplasmosis, como la opsonización de parásitos extracelulares, con la subsiguiente fagocitosis por parte de los macrófagos (Sibley et al., 1985).

1.5. Patogenia

Durante la fase de parasitemia, que puede tener su origen en una primoinfección o, más comúnmente, en una reactivación de una infección crónica durante la gestación, los taquizoítos se diseminan e invaden diferentes tejidos, siendo capaces de atravesar la placenta y llegar hasta los tejidos fetales (Dubey et al., 2006a). Se han postulado varios mecanismos por los cuales tendría lugar la muerte del feto. En primer lugar, la acción directa del parásito debido a la invasión y multiplicación en los tejidos placentarios y fetales que originaría graves lesiones que afectarían a la superviviencia fetal. A su vez, las lesiones en la placenta podrían provocar el aborto por una insuficiente oxigenación y nutrición del feto, ya sea directamente por la multiplicación de *N. caninum* en la misma o por la respuesta inmunitaria maternal de tipo inflamatorio secundaria a la infección placentaria que produciría la liberación de prostaglandinas maternales y consecuentemente luteolisis y aborto (Dubey et al., 2006a).

Por otro lado, una vez que el parásito invade placenta y feto, las diferentes consecuencias de la infección (aborto, nacimiento de terneros con signos clínicos neuromusculares, y más frecuentemente nacimiento de terneros sanos pero congénitamente infectados) van a depender de diversos factores relacionados tanto con el hospedador como con el propio parásito.

1.5.1. Inmunidad materna durante la gestación e inmunocompetencia fetal

La gestación, conduce a una situación inmunológica diferente en el animal, ya que se produce un cambio en la modulación de la respuesta inmunitaria de la madre, que tiende a ser de tipo Th2 (Lin et al., 1993). Esta respuesta inmunitaria se caracteriza por un incremento en la secreción de citoquinas reguladoras IL-4, IL-10, TGF-β que inhiben la respuesta de tipo inflamatoria mediada por IFN-γ, lo cual resulta de suma importancia para evitar un posible rechazo inmunológico del feto por parte de la madre (Innes et al., 2005). A pesar de que la respuesta inmunitaria de tipo Th1 es necesaria para el control de la multiplicación del parásito en el hospedador, se ha sugerido que, en los animales gestantes, una respuesta de tipo Th1, principalmente, a nivel de la interfase materno-fetal, podría comprometer la gestación y contribuir a la muerte del feto (Innes et al., 2002). Por otro lado, una respuesta del tipo Th2, necesaria para el mantenimiento de la gestación (Wegmann et al., 1993), podría permitir la transmisión del parásito al feto por incremento

de la carga parasitaria en los tejidos maternos (Entrican, 2002; Innes et al., 2002; Quinn et al., 2002).

En la infección experimental por *N. caninum* en modelos murinos gestantes, se ha descrito que niveles elevados de IL-4 durante la gestación pueden dar lugar a una disminución de la inmunidad mediada por células y afectar a la tasa de transmisión vertical (Quinn et al., 2004). En este sentido, Long y Baszler observaron una disminución en la transmisión vertical cuando los animales fueron infectados con una dosis subletal de *N. caninum* junto con la administración de un anticuerpo monoclonal frente a IL-4 antes de la gestación, e inoculados de nuevo con una dosis de un aislado virulento del parásito durante la gestación (Long & Baszler, 2000). Los resultados obtenidos en un estudio reciente demuestran que la respuesta inmunitaria desarrollada en la placenta tras la infección depende del período de gestación, influyendo de forma importante en la patogenia de la infección en el feto y en el periodo postnatal (López-Pérez et al., 2010; López-Pérez et al., 2011). En el primer tercio de gestacion se observó un aumento en la expresión de IFN-γ, TNF-α e IL-10 en la placenta que pudo facilitar la transmisión del parásito al feto y tener efectos tóxicos sobre la placenta y el feto (López-Pérez et al., 2011). Cuando la infección tuvo lugar en el segundo tercio se detectó un aumento en la expresión de IL-4 en la placenta que pudo favorecer la multiplicación incontrolada del parásito, y una mayor transmisión transplacentaria (López-Pérez et al., 2010). Después de la infección en el tercer tercio, se observó una reducción en la expresión de IL-4 en el bazo de las madres que podría contribuir al control de la multiplicación del parásito en los tejidos maternos, reduciendo el número de parásitos en la interfase materno-fetal (López-Pérez et al., 2011).

En el ganado bovino, la muerte fetal que tiene lugar tras la infección experimental de vacas en el primer tercio de la gestación se ha asociado a una amplia distribución del parásito junto con niveles muy elevados de citoquinas de tipo Th1 y Th2 en la interfase materno-fetal, y un infiltrado inflamatorio asociado a las lesiones, caracterizado por el incremento del número de células NK y linfocitos T $CD4^+$ y $CD8^+$ (Maley et al., 2006; Rosbottom et al., 2008). Dicha respuesta inmunitaria local no se observa cuando el feto sobrevive (Maley et al., 2006). A medida que avanza la gestación, la consecuencias de la infección pueden ser menos graves, indicando que la infección está siendo controlada, pudiendo no terminar en fallo reproductivo (Maley et al., 2003). El balance entre la respuesta de tipo Th1 y Th2 en la interfase materno-fetal y a nivel periférico, en infecciones tanto en fases tempranas como tardías de la gestación, es similar (Rosbottom et al., 2007; Rosbottom et al., 2008). Sin embargo, la magnitud de la expresión de las citoquinas es muy diferente entre ambos periodos, observándose un significativo aumento en la interfase materno-fetal de aquellos animales en los que se produce la muerte fetal (Rosbottom et al., 2008). Esto sugiere que la muerte fetal en el ganado bovino podría estar más relacionada con la intensidad de la respuesta inmunitaria que con un desequilibrio del balance Th1/Th2. No obstante, en un estudio reciente se ha observado una extensa respuesta inflamatoria en interfase materno-fetal tras la recrudescencia de una infección natural en el segundo tercio de gestación (Rosbottom et al., 2011). Los autores sugieren que dicha respuesta, caracterizada por un infiltrado inflamatorio de linfocitos T $CD4^+$ y T $CD8^+$ y

elevados niveles de expresión de IFN-γ e IL-4, lejos de afectar a la superviviencia fetal, podría contribuir al control de la parasitosis en la placenta.

Por otro lado, se sabe que la supervivencia del feto depende del estado de madurez de su sistema inmunitario y de su capacidad para hacer frente a la infección, por tanto cuánto mas temprano se produzca la infección durante la gestacion más graves serán las consecuencias (Innes et al., 2002; Collantes-Fernández et al., 2006c). En este sentido, en un feto bovino los diferentes órganos linfoides timo, bazo y ganglios linfáticos periféricos comienzan a reconocer y a responder frente a microorganismos durante el segundo tercio de la gestación (Osburn, 1988). En el primer trimestre de la gestación, el feto es excepcionalmente vulnerable a la infección por *N. caninum* y con poca probabilidad sobrevivirá (Williams et al., 2000; Innes et al., 2001). En el segundo tercio de gestación se ha observado tanto la muerte fetal (Dubey et al., 1992; Barr et al., 1994) como el nacimiento de terneros congénitamente infectados (Innes et al., 2001). En este periodo, la respuesta inmunitaria mostrada por el feto podría ser suficiente para evitar la pérdida fetal, pero no la transmisión transplacentaria (Quintanilla-Gozalo et al., 2000; Williams et al., 2009). En el tercer trimestre de gestación, el feto ya se considera inmunocompetente (Osburn et al., 1982), y podría controlar la multiplicación del parásito y limitar el desarrollo de lesiones (Innes et al., 2002; Dubey et al., 2006), permitiendo su supervivencia (Barr et al., 1994; Williams et al., 2000; Innes et al., 2001; Maley et al., 2003). En el ganado bovino infectado naturalmente, el nacimiento de terneros infectados y clínicamente sanos es la consecuencia más frecuente de la infección, por lo que se supone que la transmisión ocurriría fundamentalmente en periodos tardíos de la gestación (Quintanilla-Gozalo et al., 2000; Innes et al., 2005).

1.5.2. Aislado del parásito

El aislado de *N. caninum* con el que se produce la infección puede ser determinante en la presentación y gravedad de la enfermedad. En este sentido, se ha observado cierta diversidad biológica entre algunos de los aislados del parásito en infecciones experimentales en ratón (Lindsay et al., 1995; Atkinson et al., 1999; Pérez-Zaballos et al., 2005; Collantes-Fernández et al., 2006b) y en estudios *in vitro* (Schock et al., 2001; Pérez-Zaballos et al., 2005). Recientemente, el grupo SALUVET ha caracterizado, tanto *in vitro* como en un modelo murino de infección cerebral y congénita, un amplio número de aislados obtenidos de terneros sanos pero congénitamente infectados. Dichos estudios han demostrado diferencias en la virulencia entre aislados basadas en la distribución orgánica, cargas parasitarias y gravedad de las lesiones en cerebro (Pereira García-Melo et al., 2010), en la transmisión transplacentaria y mortalidad neonatal (Regidor-Cerrillo et al., 2010) y en la capacidad invasiva y la tasa de proliferación *in vitro* (Regidor-Cerrillo et al., 2011). La influencia de este factor en el ganado bovino se conoce poco. Hasta la fecha, en la mayoría de las infecciones experimentales se han utilizado los aislados de referencia Nc-Liverpool o Nc-1, aunque también se han empleado otros aislados en infecciones experimentales, como Nc-BPA1 o Nc-Illinois, con resultados variables dependiendo, sobre todo de otros factores, que se explican en este apartado, como la edad gestacional, la dosis infectiva o la vía de inoculación (Dubey et al., 2006).

1.5.3. Vía de inoculación y dosis infectante

La vía de inoculación es un factor que puede influir en la magnitud de las consecuencias de la infección. En infecciones experimentales se han empleado diversas vías de inoculación que, sin embargo, no representan de forma real las vías naturales de transmisión del parásito. Por tanto, no siempre es posible obtener una respuesta inmunitaria equivalente a la que se desarrolla en el curso de una infección natural. La vía intravenosa ha sido la más frecuentemente utilizada en infecciones experimentales en el ganado bovino. Dicha vía intenta simular la diseminación hematógena del parásito por el organismo tras una primoinfección o una reactivación, permitiendo probablemente la llegada del parásito a la placenta de forma más rápida y en mayor cantidad, a diferencia de la inoculación subcutánea donde el parásito se dirigirá a los linfonódulos regionales y posteriormente se diseminará por el organismo pudiendo llegar menos cantidad de parásito a la placenta. La inoculación intravenosa de una alta dosis de taquizoítos (5×10^8) del aislado Nc-1 en el día 70 de gestación, indujo una mortalidad del 100% mientras en la inoculación subcutánea la mortalidad se redujo a la mitad (Macaldowie et al., 2004). Por otro lado, la administración de ooquistes por vía oral en vacas gestantes en diferentes momentos de gestación (días 70, 120 y 210) causó tanto mortalidad fetal en el día 120 (16,6%) como transmisión congénita en el día 210 (80%) (McCann et al., 2007).

La dosis infectiva también parece ser determinante en el desarrollo de la enfermedad, asociándose por lo general una mayor dosis de parásito con incrementos en las tasas de mortalidad y presencia de lesiones graves en los modelos murinos (Long et al., 1998; Collantes-Fernández et al., 2004), probablemente debido a una mayor magnitud y/o duración de la parasitemia.

1.6. Signos clínicos

La infección por *N. caninum* en el ganado bovino no gestante es generalmente asintomática, mientras que en animales gestantes tiene como sígno clínico más relevante el aborto (Dubey, 2005). El aborto puede ocurrir a partir del tercer mes de gestación aunque suele observarse con más frecuencia entre los 5 y 6 meses (Dubey & Lindsay, 1996; Dubey, 1999a). Si la infección ocurre en el primer tercio de la gestación, el feto suele ser reabsorbido y lo que se observa clínicamente es una repetición del celo. Si la muerte se produce entre los 3 y 8 meses de gestación, el feto suele ser eliminado presentando una autolisis moderada. Sin embargo, algunos fetos que mueren antes del quinto mes podrían momificarse y quedar retenidos en el útero durante meses (Dubey, 2005). Si la infección *in utero* ocurre en gestaciones más avanzadas, a partir del quinto mes de gestación, disminuye el riesgo de muerte fetal y el signo más frequente será el nacimiento de terneros sanos pero congénitamente infectados, que presentarán altos títulos de anticuerpos precalostrales (Quintanilla-Gozalo et al., 2000; Williams et al., 2000). En un número muy reducido de casos pueden nacer terneros infectados muy débiles con signos clínicos neurológicos, que van desde una ligera incoordinación hasta una parálisis completa, y en los casos más graves se puede observar malformaciones en la espina dorsal, estrechamiento de la médula espinal, hidrocefalia y neumonía (Dubey et al., 1992; Bryan et al., 1994;

Gunning et al., 1994). Dichos signos aparecen en la primera semana después del parto, aunque estos pueden retrasarse hasta transcurridas dos semanas del nacimiento (Dubey & de Lahunta, 1993; Duivenvoorden & Lusis, 1995), y suelen empeorar hasta la total parálisis y muerte del animal durante el primer mes de vida (Dubey et al., 1992; Gunning et al., 1994; Dubey et al., 2006).

A nivel de rebaño, los abortos pueden responder a tres patrones: epidémico, endémico o esporádico (Thurmond & Hietala, 1997a; Dubey, 1999b). Por otro lado, no parece que la infección por *N. caninum* afecte a otros aspectos de la reproducción del ganado bovino. En un estudio reciente, el intervalo entre el aborto y la primera inseminación artificial, el número de inseminaciones necesarias para quedar gestante y el intervalo entre el aborto y la siguiente gestación fueron menores en vacas abortadas infectadas por *N. caninum* que en vacas abortadas y no infectadas (Santolaria et al., 2009).

1.7. Lesiones

Las lesiones asociadas a la infección por *N. caninum* se localizan principalmente en la placenta y en el feto. En general, las lesiones son de naturaleza inflamatoria no supurativa. En la placenta se suelen observar focos de necrosis y zonas de intensa inflamación con infiltración de células mononucleares, que en procesos avanzados pueden progresar hacia la regeneración conjuntiva con fibrosis e incluso calcificación de los focos necróticos (Barr et al., 1994; Maley et al., 2003). Estas lesiones placentarias son más graves y la necrosis más amplia cuando se ha producido muerte fetal que cuando el feto sobrevive, lo cual es más frecuente en las fases tempranas de la gestación (Gibney et al., 2008). En fetos abortados es frecuente observar una encefalitis multifocal necrótica no purulenta con manguitos perivasculares, microgliosis y presencia de astrocitos, pudiendo existir zonas centrales de necrosis e, incluso, mineralización (Barr et al., 1991a; Dubey & Lindsay, 1996), junto con miocarditis y hepatitis difusa (Barr et al., 1993; Collantes-Fernández et al., 2006c). En fetos inmunocompetentes, la multiplicación del parásito es más restringida, observándose necrosis focal, rodeada por un intenso infiltrado inflamatorio que contiene microglia, astrocitos y células de la serie linfoide monocítica (Barr et al., 1994; Otter et al., 1995; Wouda et al., 1997; Schock et al., 2000). Estos focos pueden calcificarse más tarde (Boulton et al., 1995; González et al., 1999). Las lesiones producidas por *N. caninum* son más graves en los fetos abortados en el primer y segundo tercio de la gestación que en aquellos abortados al final de la gestación (Collantes-Fernández et al., 2006c), y en aquellos asociados a abortos con patrón epidémico que en los procedentes de abortos con patrón endémico (Wouda et al., 1997; Collantes-Fernández et al., 2006a). En los terneros congénitamente infectados sin signos clínicos y en animales adultos la presencia de lesiones es rara, restringiéndose principalmente al sistema nervioso central (Barr et al., 1991b; Bryan et al., 1994; Sawada et al., 2000).

1.8. Diagnóstico

El diagnóstico de la neosporosis bovina es complejo y ha de realizarse de manera ordenada y sistemática. La selección del protocolo diagnóstico dependerá de la información que se pretende conseguir. Se puede seguir un protocolo de diagnóstico individual, para conocer la causa del aborto en una reproductora o bien seguir un protocolo de diagnóstico colectivo, cuando lo que interesa es conocer la situación de la infección en una explotación, la implicación de *N. caninum* en el problema reproductivo de la misma o el principal modo de transmisión del parásito con el fin de establecer las medidas de control apropiadas en cada caso.

Actualmente, existe una amplia batería de técnicas diagnósticas pero no todas ofrecen la misma información o tienen la misma fiabilidad. En la práctica, el diagnóstico se debe abordar desde un punto de vista colectivo. La herramienta de elección para conocer la situación inicial de la granja es la detección de anticuerpos específicos frente a *N. caninum* en muestras de suero sanguíneo. Las técnicas serológicas más útiles incluyen la detección de anticuerpos por inmunofluorescencia (IFI) y los ensayos inmunoenzimáticos (ELISAs) (Bjorkman & Uggla, 1999), utilizando el inmunoblot como prueba confirmatoria en casos dudosos (Álvarez-García et al., 2003; von Blumroder et al., 2004). La detección de anticuerpos en muestras de leche mediante ELISA es una herramienta igualmente factible con resultados equiparables a los de suero, con las ventajas añadidas de menores costes y manejo del animal (Bjorkman et al., 1997; Salas-Calvo et al., 2005). Sin embargo, debido al efecto de dilución de los anticuerpos en el tanque, únicamente es útil en rebaños con prevalencias intra-rebaño de la infección superiores al 10-15%. El uso de esta técnica puede ser útil tanto para conocer el estado serológico inicial de una granja como para realizar el seguimiento de un programa de control de una manera económica y fiable.

Las seroprevalencias de la explotación y de las vacas abortadas permiten determinar si el problema de abortos está asociado a la infección por *N. caninum* y la magnitud de éste. Este primer abordaje serológico, sobre todo en los casos de aparición de brotes inesperados de abortos, se debe acompañar de la aplicación de técnicas de detección del parásito en los tejidos de fetos abortados (cerebro, placenta y corazón principalmente), mediante la técnica de PCR y de la determinación de la existencia de lesiones compatibles en dichos tejidos. El diagnóstico de infección fetal por *Neospora* también se complementa con la detección de anticuerpos específicos en fluidos de fetos con más de 5 meses de gestación (Pereira-Bueno et al., 2003; Dubey & Schares, 2006).

Una vez confirmadas la infección por *N. caninum* y la situación serológica de la granja, el siguiente paso es conocer la fase de la infección en la que se encuentran los animales y el principal modo de transmisión en la granja. Esta información será de utilidad para la elección de las medidas de control más eficaces frente a la infección por *N. caninum*. La relación entre la serología materna y la de la descendencia, así como la distribución de los animales seropositivos por edades nos orientará sobre el modo de transmisión predominante en esa granja. Además, la utilización de herramientas diagnósticas

como el ELISA de avidez puede ser de gran utilidad (Aguado-Martínez et al., 2005). Esta técnica permite detectar los anticuerpos de baja afinidad que aparecen en las fases iniciales de la infección, permitiendo discriminar entre explotaciones en las que gran parte de los animales presentan una infección reciente como causa de la transmisión horizontal, de aquellas en las que sus animales están crónicamente infectados. Los nuevos avances llevados a cabo en el campo del diagnóstico plantean la posibilidad de la utilización de técnicas serológicas de nueva generación, como el uso de ELISAs basados en proteínas recombinantes del parásito específicas de diferentes estadios del mismo (Aguado-Martínez et al., 2008), que aportan una información más completa sobre la fase de la infección en la que se encuentran los animales y, por tanto, el modo de transmisión predominante.

1.9. Control

La situación epidemiológica y el impacto económico de la neosporosis bovina varían en cada país, región e incluso en cada explotación (Dubey et al., 2007). Como se ha señalado anteriormente, para la puesta en marcha de un programa de control de la enfermedad en el rebaño es necesaria la realización de un protocolo diagnóstico que permita conocer la situación epidemiológica de la que se parte. En la actualidad, la única opción existente para el control de la infección en el ganado se basa principalmente en la implementación de medidas de manejo (Figura 2). La quimioterapia e inmunoprofilaxis, actualmente bajo estudio, se presentan como alternativas potenciales.

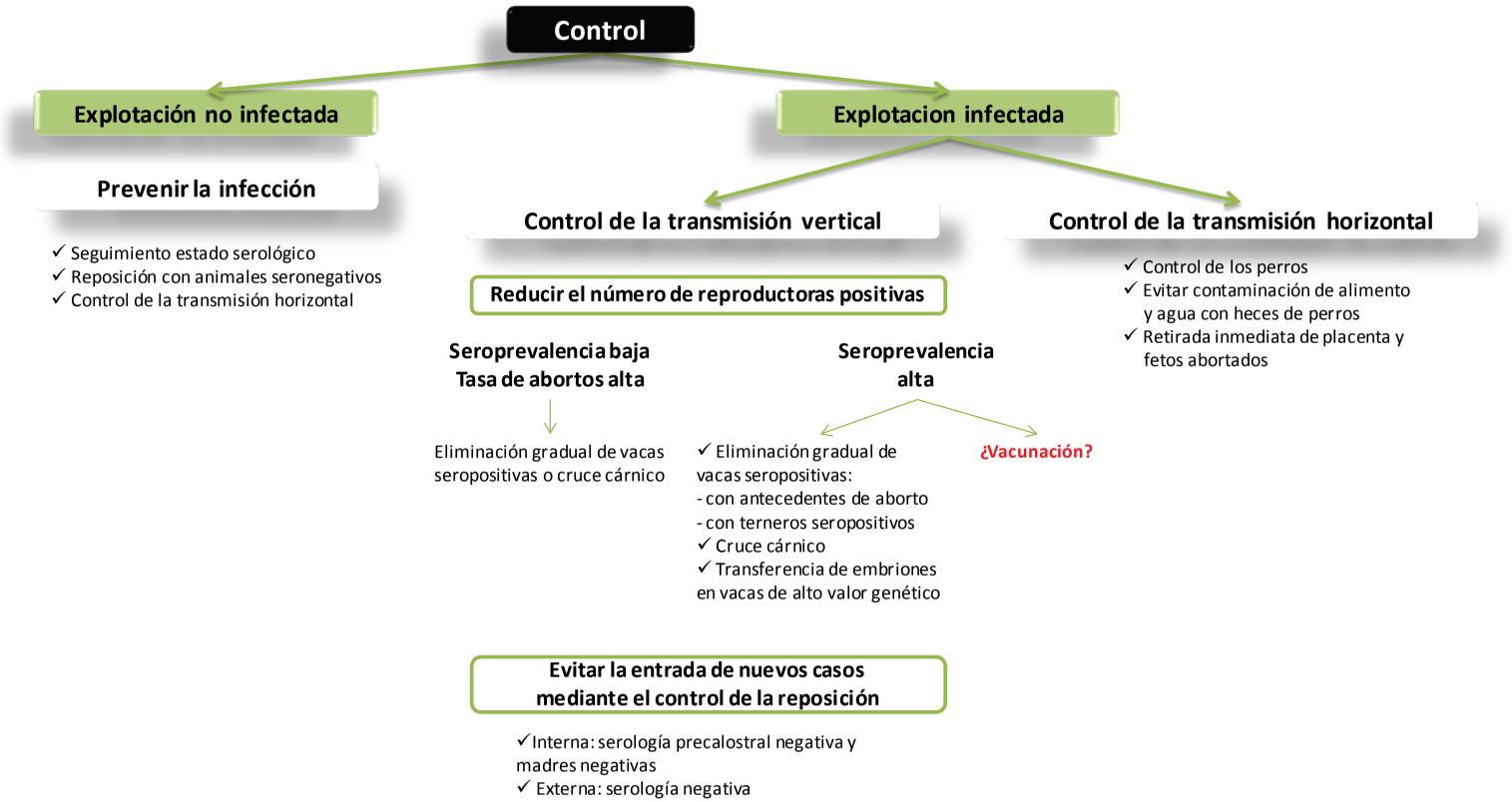


Figura 2. Principales medidas de control frente a la infección por *N.caninum* en las explotaciones bovinas

1.9.1. Medidas de manejo

La identificación de los diferentes factores de riesgo asociados a la infección o a la presencia de aborto dentro de un rebaño es necesaria para implementar un programa de control de la enfermedad que permita corregir determinadas prácticas con el ganado. En este sentido, diversos estudios epidemiológicos de tipo caso-control o transversales han permitido la identificación de numerosos factores de riesgo tanto a nivel individual como de rebaño (Dubey et al., 2007; Dubey & Schares, 2011). Respecto a los factores de riesgo asociados a la infección por *N. caninum,* la presencia de perros en la granja, el número en que se encuentran y sus hábitos alimenticios, basados en la ingestión de placentas y fetos abortados, aumentan las posibilidades de que el ganado se infecte (Dubey et al., 2007; Vanleeuwen et al., 2010). El riesgo de infección también puede aumentar con la edad de los animales y el tipo de manejo del rebaño (ej. alimentación, densidad, alojamientos) (Dubey et al., 2007; Moore et al., 2009). De entre los factores de riesgo asociados al aborto, destacan tanto la seropositividad individual como la seroprevalencia de rebaño. Por otro lado, los factores de riesgo asociados al aborto variarán según se trate de abortos de origen epidémico o endémico. Así, la edad de los animales y la presencia y número de perros están relacionados positivamente con los abortos de tipo epidémico (Dubey et al., 2007). Por tanto, los diferentes programas de control se basarán principalmente en la implementación de medidas de manejo y bioseguridad para reducir la exposición de los animales a todos estos factores de riesgo. La elección de dichas medidas dependerá, fundamentalmente, del objetivo que se plantee, ya sea prevenir la entrada de la infección en granjas libres o bien controlar la infección, reduciendo la prevalencia en las explotaciones infectadas y su propagación a otras.

1.9.1.1. Prevención de la infección

Cuando se pretenda evitar la entrada de la infección en la granja, se utilizarán medidas de bioseguridad y manejo como:

i. Seguimiento serológico de los animales con el fin de detectar nuevos casos y eliminarlos.

ii. Política de adquisición y reposición con animales que presenten diagnóstico negativo.

iii. Control de la transmisión horizontal, adoptando las medidas para evitar la infección postnatal que describiremos posteriormente.

1.9.1.2. Control de la transmisión vertical

Debido a la importancia de la transmisión vertical en el mantenimiento de la infección dentro de un rebaño, las medidas encaminadas a evitarla deben constituir la base del control de esta enfermedad. Dichas medidas engloban:

i. El diagnóstico de todas las reproductoras que vayan a formar parte de la reposición, seleccionando para la renovación sólo aquellos animales libres de la infección. Tanto si la reposición de las reproductoras es externa como interna se deberán seleccionar

animales libres de la infección mediante análisis serológico. Si fuera posible, lo adecuado es realizar análisis serológico de una muestra de suero obtenida del ternero antes de la primera toma de calostro. Si no es posible, lo recomendable es realizar dos análisis diferentes separados 4-6 semanas para confirmar la seronegatividad a partir de los 6 meses de edad y, por otro lado, elegir individuos que provengan de madres seronegativas. En el caso de reposición externa, además se debería garantizar que la explotación de origen no tiene historial de abortos asociados a la infección por *N. caninum*.

ii. El seguimiento del estado serológico de las reproductoras y sacrificio selectivo de hembras seropositivas. Esta medida es, hasta la fecha, la más eficaz en el control de la neosporosis en la granja (Thurmond & Hietala, 1995; French et al., 1999; Jensen et al., 1999). En la actualidad, existen diversas estrategias de diagnóstico y sacrificio selectivo, encaminadas a la reducción/eliminación de la infección en las explotaciones. La selección de una estrategia u otra depende de la seroprevalencia en la granja y se llevará a cabo tras un análisis de coste-beneficio (Reichel & Ellis, 2006). Su instauración puede hacerse de dos formas: drásticamente, eliminando todas las vacas seropositivas, o gradualmente. Si la seroprevalencia es baja puede ser factible la eliminación gradual de las vacas positivas. Si por el contrario ésta es elevada es muy probable que la eliminación de los animales seropositivos no sea una medida viable económicamente y debamos actuar siguiendo pautas más moderadas como la eliminación de vacas seropositivas o seropositivas con antecedentes de aborto, la inseminación de la progenie de vacas seropositivas con semen de toros de aptitud cárnica o evitar la reposición con crías de hembras seropositivas.

iii. La transferencia de embriones de hembras infectadas a no infectadas. Esta medida adquiere gran importancia en el caso de reproductoras de alto valor genético (Baillargeon et al., 2001) y se propone con el objetivo de reducir el riesgo de TTEn.

iv. El mantenimiento de un buen estado higiénico-sanitario y de bienestar de los animales para evitar posibles estados de enfermedad o estrés que sean el origen de una inmunosupresión con la consiguiente recrudescencia de la neosporosis en animales crónicamente infectados por *N. caninum* (Dubey et al., 2007).

1.9.1.3. Control de la transmisión horizontal

Entre las medidas específicas que podrían contemplarse para el control de la transmisión horizontal se encuentran las medidas higiénicas encaminadas a reducir la posible contaminación ambiental con fases de resistencia del parásito (Dubey et al., 2007; Dubey & Schares, 2011), como:

i. El control de la población canina y otros hospedadores definitivos, evitando en la medida de lo posible el acceso de éstos al pasto y pienso, reduciendo así el riesgo de que los animales se infecten vía oral con ooquistes.

ii. La eliminación de los fetos abortados y tejidos placentarios para disminuir el riesgo de infección en el hospedador definitivo después de la ingestión de tejidos bovinos infectados con *N. caninum*.

iii. La implementación de medidas de bioseguridad que impidan el contacto de los perros con animales silvestres, que puedan actuar como hospedadores intermediarios o reservorios de la infección para el hospedador definitivo.

1.9.2. Quimioterapia y quimioprofilaxis

En la actualidad, el control farmacológico de la neosporosis bovina parece inviable por la falta de fármacos realmente eficaces y económicamente rentables (Dubey & Schares, 2011). Además, las cuestiones referentes a los costes y a los residuos generados por estos fármacos en la carne y la leche deben ser analizadas, pudiendo limitar la quimioterapia como medida de control (Reichel & Ellis, 2006). Hasta la fecha se han analizado *in vitro* un gran número de fármacos para determinar su eficacia en el tratamiento frente al parásito. Diversos estudios experimentales sugieren que el tratamiento con ciertos coccidiostáticos como las sulfadíazinas podría constituir una opción para el control de la neosporosis (Kritzner et al., 2002; Gottstein et al., 2005). En los últimos años, el toltrazuril ha sido el fármaco más estudiado. En el modelo murino gestante, se han observado eficacias parciales en cuanto a morbilidad, mortalidad o transmisión vertical tras el tratamiento con toltrazuril en las madres (Gottstein et al., 2005) o en las crías (Strohbusch et al., 2009a). También se ha encontrado un cierto efecto del toltrazuril y su derivado ponazuril tanto *in vitro* como *in vivo* en terneros tratados, en los que se detectó la disminución de la presencia del parásito en cerebro y otros tejidos poco tiempo después de la infección (Strohbusch et al., 2009a). Sin embargo, no existe información sobre el efecto de estos fármacos en infecciones naturales en el ganado bovino.

Por tanto, el uso de tratamientos profilácticos, aunque ha sido ampliamente considerado como herramienta para el control, plantea grandes inconvenientes, favoreciendo que otras herramientas como la inmunoprofilaxis aparezca como una alternativa para el control de este tipo de enfermedades.

2. LA VACUNACIÓN FRENTE A LA NEOSPOROSIS BOVINA

Diversos estudios epidemiológicos y de infecciones experimentales sugieren que el ganado bovino puede desarrollar cierto grado de inmunidad protectora frente a la infección. En este sentido, McAllister et al. (2000) observaron que tras la exposición al parásito, las vacas persistentemente infectadas tenían una menor probabilidad de abortar que las vacas primoinfectadas. Asimismo, el riesgo de aborto y de transmisión vertical asociado a la infección fue más elevado en la primera gestación que en las posteriores (Thurmond & Hietala, 1997a; Hernández et al., 2002). A nivel experimental, la infección en el primer tercio de gestación en novillas crónicamente infectadas no indujo pérdida fetal, por el contrario, novillas primoinfectadas en el mismo momento de gestación presentaron aborto (Williams et al., 2003). Todo esto indica la existencia de cierta inmunidad adquirida ante la infección que, junto con la falta de medidas de control económicamente viables para el control de la enfermedad, han llevado a que en los últimos años se esté trabajando intensamente en el campo del desarrollo de vacunas frente a la neosporosis bovina.

En la actualidad no existe ninguna vacuna en el mercado, y sólo una vacuna inactivada (NeoGuard®, Intervet) estuvo aprobada para su uso en vacas gestantes en los Estados Unidos hasta el año 2009. A pesar de ser una vacuna segura, su papel protector frente al aborto fue muy discutido, ya que esta vacuna no confería protección en vacas gestantes infectadas experimentalmente con el parásito (Andrianarivo et al., 2000), y en varios estudios de campo presentó una eficacia variable (5,2%-54%) (Heuer et al., 2003; Romero et al., 2004; Weston et al., 2011). Hasta la fecha, los resultados más prometedores de protección frente al aborto han sido observados tras el empleo de vacunas vivas atenuadas en infecciones experimentales en el ganado bovino (Guy et al., 2005; Williams et al., 2007). Finalmente, las investigaciones más recientes en el campo de la inmunoprofilaxis de la neosporosis también se están dirigiendo hacia el diseño de vacunas de nueva generación empleando diversos antígenos recombinantes, parásitos transgénicos o nuevas técnicas de adyuvantación (Innes et al., 2011).

2.1. La vacunación como alternativa rentable

La magnitud de las pérdidas económicas asociadas a la neosporosis bovina se ha estimado en cientos de millones de euros por año en todo el mundo (Dubey et al., 2007). Dichas pérdidas incluyen unos costes directos, asociados al aborto (Thornton et al., 1994; Pfeiffer et al., 2002) y unos costes indirectos, debidos al incremento de la reposición (Thurmond & Hietala, 1996; Tiwari et al., 2005; Bartels et al., 2006), la posible disminución de la producción de leche (Thurmond & Hietala, 1997b; Hernández et al., 2001; Hobson et al., 2002), la reducción del valor de la recría (Trees et al., 1999), la asistencia veterinaria y los gastos de diagnóstico.

Numerosos estudios económicos basados en modelos predictivos ofrecen estimaciones de las pérdidas económicas, y han promovido y desarrollado estrategias de control de la neosporosis bovina, basadas fundamentalmente en cálculos de coste-beneficio (French et al., 1999; Larson et al., 2004; Hasler et al., 2006a; Hasler et al., 2006b; Hasler et al., 2008). Entre las medidas de control, la vacunación se ha sugerido como una de las principales alternativas de futuro en el control de esta enfermedad que debería complementar a las medidas de manejo del rebaño indicadas anteriormente (Reichel & Ellis, 2006). En dichos estudios se evaluó la relación coste/beneficio de diferentes estrategias de control, utilizando datos epidemiológicos extraídos de la literatura sobre la situación de la neosporosis en Australia y Nueva Zelanda. Los resultados indicaron que en rebaños con elevada prevalencia de infección (>21%), la opción más rentable era la vacunación. No realizar ningún programa de control aparecía como la segunda alternativa más rentable en el caso de prevalencias más bajas (<21%), mientras que realizar diagnóstico y sacrificio o la aplicación de un tratamiento terapeútico, aún siendo las más efectivas para el control de la infección, se presentaban como las menos viables económicamente (Figura 3). En otro trabajo, los autores sugieren que los límites de intervención rentables se sitúan en unas prevalencias intra-rebaño entre el 10-15% (Reichel & Ellis, 2008). Sin embargo, en el supuesto de que hubiera una vacuna eficaz y segura disponible en el mercado podría ser rentable acometer programas de control con prevalencias intra-rebaño del 3%.

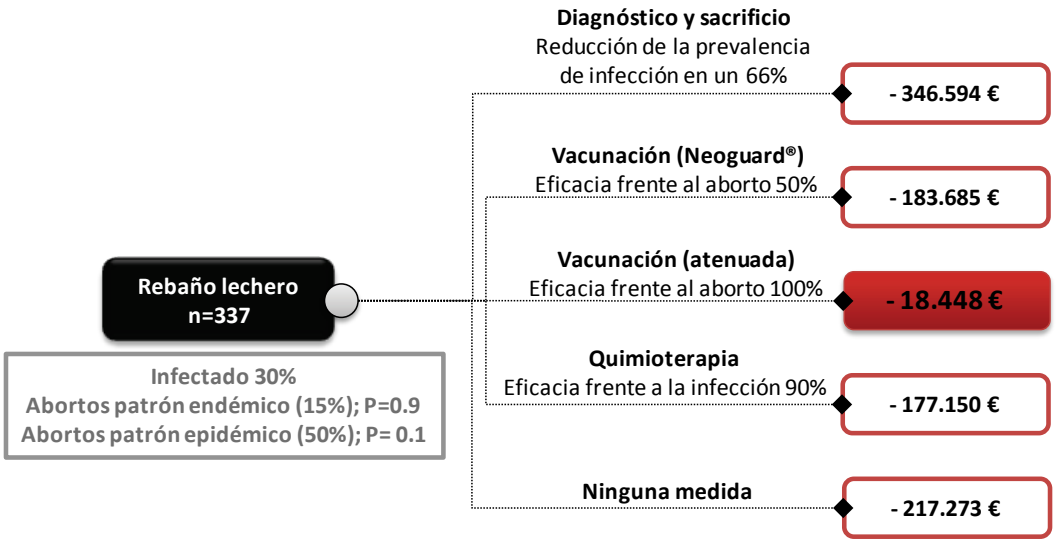


Figura 3. Análisis del coste de varias estrategias de control de la infección por *N. caninum* en un periodo de 5 años en una granja tipo de leche de Nueva Zelanda (modificado de Reichel & Ellis, 2009). Tras el análisis mediante árboles de decisión, la vacunación con un aislado atenuado (Nc-Nowra; Miller et al., 2002) se ha estimado como la medida más rentable para el control de la infección dentro del rebaño.

2.2. Diseño de vacunas frente a la infección por *N. caninum* y comparación con otras protozoosis

La complejidad del ciclo biológico de los parásitos apicomplejos formadores de quistes, como *N. caninum,* exige el conocimiento de la biología del parásito, la interacción parásito-hospedador, los diferentes estadios parasitarios, los antígenos involucrados en la supervivencia del parásito en el hospedador y los componentes de la respuesta inmunitaria implicada en la protección, a fin de desarrollar eficazmente una estrategia de vacunación (Innes & Vermeulen, 2006). Por ello, durante el diseño de una vacuna frente a estos parásitos, y en general en la mayoría de las vacunas, hay que considerar múltiples factores que determinarán el éxito o el fracaso de una formulación. El primer paso es la selección, caracterización, purificación y síntesis, cuando corresponda, de los componentes que confieren inmunidad a la vacuna, los antígenos. Estos componentes formarán la base del diseño de una vacuna. Por otro lado, la utilización de sustancias que aumenten la potencia inmunogénica de las vacunas, como adjuvantes o inmunoestimuladores, es un factor clave en el desarrollo de algunas vacunas. La vía de administración o la dosis pueden ser variables que determinen la eficacia e inocuidad de una formulación. Por último, los ensayos en modelos experimentales, extrapolables a los modelos naturales de la enfermedad, van a adquirir especial importancia como estrategia de cribado de los diferentes formulaciones diseñadas.

2.2.1. Factores que influencian la eficacia inmunoprotectora de una vacuna

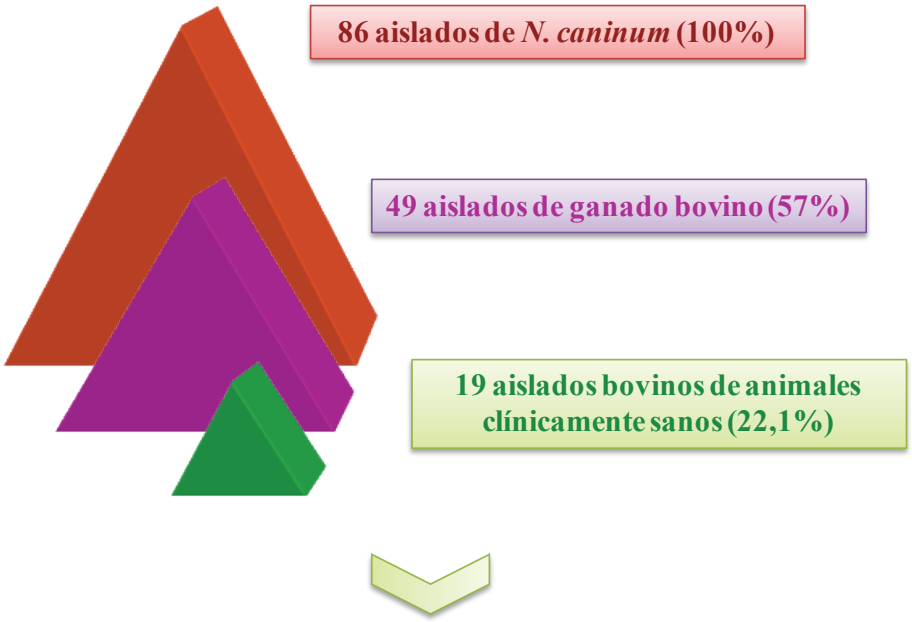
2.2.1.1. Composición antigénica

2.2.1.1.1. Diversidad biológica

El conocimiento de la diversidad genética intra-específica y cómo esta puede influir en la epidemiología y patogenia de la enfermedad es un punto clave para el desarrollo de estrategias de vacunación. Los problemas en la eficacia de las vacunaciones debidas a la variabilidad antigénica entre especies y cepas, han sido descritos en la coccidiosis aviar, sobre todo en la utilización de vacunas vivas (Smith et al., 2002). Por ello, ya existen vacunas comerciales que incluyen cepas o especies de *Eimeria* antigénicamente diferentes (Vermeulen et al., 2001).

En los protozoos apicomplejos formadores de quistes, la influencia de este factor sobre la protección no es tan clara. Históricamente, la mayoría de los aislados de *T. gondii* que se conocían estaban englobados en tres linajes, encontrándose una clara asociación entre éstos y la virulencia. Recientemente, se ha demostrado que la biodiversidad y la distribución geográfica de los diferentes aislados de *T. gondii* es mucho más amplia de lo que se pensó inicialmente (Beck et al., 2009; Dubey & Su, 2009). Sin embargo, parece poco probable que los diferentes aislados generen una inmunidad específica (Smith & Frenkel, 2003), de manera que la inmunización con diferentes aislados de *T. gondii* es capaz de generar protección cruzada ante desafíos con aislados heterólogos (Frenkel & Smith, 1982; Freyre et al., 2007).

En *N. caninum,* la amplia distribución geográfica y espectro de hospedadores y los procesos de recombinación sexual en el hospedador definitivo sugieren también una amplia diversidad genética y biológica. Desde que en 1988 se obtuviera el primer aislado de *N. caninum* (Dubey et al., 1988b), el número de aislados referenciados en la bibliografía es reducido (86 aislados). Esto se debe principalmente a que el proceso de aislamiento del parásito a partir de tejidos infectados es difícil por el bajo número y viabilidad de los parásitos presentes en la muestra (Dubey et al., 2007). Los aislados obtenidos hasta la fecha proceden de diferentes especies de hospedadores como el ganado bovino, ovino, perro, ciervo de cola blanca, búfalo de agua y bisonte europeo, siendo cerca de 50 los de origen bovino. Los aislados bovinos proceden en su mayoría de animales clínicamente afectados (Dubey et al., 2007), y sólo un número reducido de animales sanos, principalmente terneros infectados congénitamente pero clínicamente sanos (Regidor-Cerrillo et al., 2008) (Figura 4).



País	Nombre	Origen	Caracterización en un modelo murino gestante[a]	Referencia
Australia	Nc-Nowra	Ternero, 7 dias	87%	Miller et al., 2002
Brasil	Nc-Goias 1	Ternero, 4 meses	ND	Pereira Garcia-Melo et al., 2009
Eslovaquia	Nc-SKB1	Vaca adulta	ND	Reiterova et al., 2011
España	Nc-Spain2H	Ternero, 2 dias	20,4%	Regidor-Cerrillo et al., 2008, 2010
	Nc-Spain3H	Ternero, 52 dias	7%	
	Nc-Spain4H	Ternero, 22 dias	100%	
	Nc-Spain5H	Ternero, 14 dias	96%	
	Nc-Spain6	Ternero, 30 dias	29,8%	
	Nc-Spain7	Ternero, 57 dias	95%	
	Nc-Spain8	Ternero, 2 dias	1,1%	
	Nc-Spain9	Ternero, 7 dias	17,9%	
	Nc-Spain10	Ternero, 2 dias	8%	
Italia	Nc-PVI	Ternero, 45 dias	ND	Magnino et al., 1999, 2000
	Nc-PGI	Ternero, 8 meses	ND	Fioretti et al., 2000
Japón	BT-2	Ternero, 22 dias	ND	Yamane et al., 1998
	BT-3	Vaca adulta	ND	Sawada et al., 2000
Nueva Zelanda	NcNZ 1	Vaca adulta	ND	Okeoma et al., 2004
	NcNZ 2	Ternero, 2 dias		
Polonia	Nc-PolB1	Ternero, 1 dia	ND	Gozdzik and Cabaj, 2007

[a] Porcentaje de transmisión vertical del aislado en estudio.
ND: no determinado

Figura 4. Aislados de *N. caninum* de origen bovino obtenidos a partir de animales infectados pero clínicamente sanos.

Los primeros trabajos destinados al estudio de la variabilidad genética entre aislados de *N. caninum* se basaron en el análisis de la secuencia de la región ITS-1, de secuencias altamente polimórficas en *T. gondii* o bien en el empleo de la técnica RAPD (*Randomly Amplified Polymorphic DNA analysis*), evidenciándose una alta homología genética (Beck et al., 2009). Recientemente, el estudio de secuencias microsatélites polimórficas en el genoma de *N. caninum* (Regidor-Cerrillo et al., 2006), así como su aplicación en muestras biológicas (Pedraza-Díaz et al., 2009), ha permitido la detección de una amplia diversidad intra-específica, resultando ser una interesante herramienta molecular por su elevada capacidad para establecer diferencias genéticas entre aislados (Regidor-Cerrillo et al., 2006; Regidor-Cerrillo et al., 2008; Basso et al., 2009; Basso et al., 2010).

A nivel antigénico, el estudio de los perfiles antigénicos de diferentes aislados ha revelado una escasa variabilidad intra-específica de *N. caninum*. El análisis de diferentes aislados mediante la utilización de Western blotting mostró perfiles similares entre los aislados comparados (Schock et al., 2001; Miller et al., 2002). También se han realizado comparaciones a nivel proteómico entre aislados que mostraron distinta patogenicidad *in vivo* e *in vitro* a nivel biológico, detectándose variaciones a nivel proteómico y antigénico (Shin et al., 2005; Regidor-Cerrillo et al., en revisión).

A nivel biológico, la información disponible sobre la diversidad intra-específica de *N. caninum* y como podría relacionarse ésta con la patogenicidad de los diferentes aislados es escasa. Diversos autores han descrito diferencias en la capacidad de invasión y la velocidad de crecimiento *in vitro* (Schock et al., 2001; Pérez-Zaballos et al., 2005; Regidor-Cerrillo et al., 2010), así como diferencias en el comportamiento *in vivo* de diferentes aislados de *N. caninum* mediante el empleo de modelos experimentales murinos (Atkinson et al., 1999; Shibahara et al., 1999; Miller et al., 2002; Collantes-Fernández et al., 2006b). Se ha observado una mayor patogenicidad en determinados aislados, como el Nc-Liverpool, asociada a una alta morbilidad y mortalidad, importantes lesiones, así como cargas parasitarias más altas en cerebro comparado con la infección con los aislados Nc-1, Nc-SweB1, JPA1 o Nc-Nowra.

Con el fin de investigar si la obtención a partir de animales sanos de algunos aislados se relaciona con una menor patogenicidad, como ocurre con el aislado Nc-Nowra (Miller et al., 2002), el grupo SALUVET ha realizado recientemente varios estudios comparativos entre aislados de origen bovino obtenidos a partir de muestras de cerebro de terneros infectados congénitamente clínicamente sanos. Dichos aislados se caracterizaron en un modelo murino cerebral (Collantes-Fernández et al., 2006b), mostrando diferencias en distribución orgánica, cargas parasitarias y gravedad de las lesiones en cerebro (Pereira García-Melo et al., 2010). Cuando éstos se caracterizaron en un modelo murino gestante (López-Pérez et al., 2006; López-Pérez et al., 2008), nuevamente se observaron diferencias en el comportamiento, que permitieron la clasificación de los aislados en diversas categorías en función de las tasas de mortalidad neonatal y transmisión vertical: virulencia alta (90-100% de transmisión y de mortalidad post-parto), media (entre 50 y 70% de transmisión y 20-30 % de mortalidad) y baja (con un 50 % de transmisión y una mortalidad menor del 10%) (Regidor-Cerrillo et al., 2010). Estas diferencias en virulencia detec-

tadas en un modelo murino gestante se ha relacionado con la capacidad invasiva y la tasa de proliferación de estos aislados (Regidor-Cerrillo et al., 2011). Además, el uso de técnicas de resolución en gel, como el 2D DIGE (*Diferencial Gel Eelectrophoresis*) han permitido realizar el estudio proteómico comparativo entre algunos de estos aislados de alta y baja virulencia de *N. caninum*, a partir del cual ha sido posible la identificación de una serie de proteínas, algunas de las cuales parecen estar implicadas en procesos de invasión e incluso en virulencia (Regidor-Cerrillo et al., en revisión), y que por tanto podrían tener importancia inmunoprotectora.

Los resultados de todos estos trabajos muestran la variabilidad intra-específica de *N. caninum* que, sin embargo, no parece generar una inmunidad específica, pudiendo inducir una inmunidad cruzada ante desafíos heterólogos, como ya han demostrado varios trabajos de vacunación (Jenkins et al., 2004; Miller et al., 2005; Williams et al., 2007). No obstante, gran parte de los estudios de vacunas vivas e inactivadas frente a la neosporosis han utilizado desafíos homólogos. Estas aproximaciones experimentales pueden distar de lo que ocurre en las granjas con infecciones naturales, donde circulan diferentes aislados con diferentes comportamientos biológicos. Por tanto, asegurar una protección heteróloga es de sumo interés y para ello, la obtención y caracterización de un amplio panel de aislados, representativo de las diferentes poblaciones naturales, podría ser de gran utilidad. A partir de este panel se podrían seleccionar antígenos para su uso en vacunas en base a posibles factores de virulencia, atenuación o capacidad de conversión entre estadios, entre otros, de los diferentes aislados.

2.2.1.1.2. Estadio parasitario y fase de la infección

La selección de antígenos específicos de estadio que protejan frente a las diferentes fases de la infección por *N. caninum* es un punto clave en el diseño de cualquier estrategia de vacunación. En el diseño de vacunas frente a la toxoplasmosis, se han ensayado formulaciones fundamentalmente basadas en antígenos de taquizoítos, ya sean enteros vivos, inactivados o en forma de proteínas inmunodominantes de superficie, ampliamente presentes en este estadio (Buxton et al., 1993; Angus et al., 2000). La vacunación con antígenos específicos de taquizoíto tiene como finalidad proteger frente a la fase aguda de la infección, intentando limitar la parasitemia y la posterior diseminación del parásito a los tejidos, evitando la invasión de placenta y tejidos fetales que conduciría al aborto.

Por el contrario, la protección frente a la fase crónica se ha ensayado mediante la utilización de antígenos específicos de bradizoíto de *T. gondii.* La utilización de dichos antígenos tiene como objetivo el inducir una respuesta protectora frente a la adquisición de la infección por medio del consumo de quistes tisulares, la formación de quistes en cerebro o la reactivación de infecciones cerebrales con la diferenciación de bradizoítos a taquizoítos. En este sentido, la inmunización con una proteína presente en quistes tisulares con bradizoítos, MAG1, indujo una reducción en el número de quistes en el cerebro (Parmley et al., 2002).

Por otro lado, se ha probado la inmunización con una formulación que incluía múltiples antígenos recombinantes de ambos estadios de la fase asexual, obteniendo cier-

ta protección ya que disminuyó el número de quistes en el cerebro de ratones inmunizados (Vercammen et al., 2000).

En la neosporosis, la mayoría de formulaciones que se han probado hasta la fecha, se han basado principalmente en la utilización de antígenos procedentes del taquizoíto, tanto zoítos enteros, vivos o inactivados, como fracciones antigénicas (Reichel & Ellis, 2009). Esto, probablemente se debe a la mayor facilidad para la obtención de este estadio mediante técnicas de cultivo *in vitro* en comparación con el estadio de bradizoíto. Otra estrategia ampliamente utilizada ha sido la identificación y selección de antígenos recombinantes, específicos de taquizoíto o compartidos entre ambos estadios de la fase asexual, implicados en procesos de adhesión, invasión y proliferación intracelular (Hemphill et al., 2006; Innes & Vermeulen, 2006). Recientemente, se ha examinado, por primera vez, el papel de una proteína específica de bradizoíto, NcSAG4 (Fernández-García et al., 2006) en la protección frente a la infección en un modelo murino gestante, no resultando eficaz en la protección frente a la transmisión vertical (Aguado-Martínez et al., 2009). Sin embargo, el aumento del tiempo de supervivencia medio de las camadas vacunadas con esta proteína frente a las camadas no vacunadas, supone un resultado prometedor para la utilización de antígenos específicos de bradizoíto.

2.2.1.1.3. Dosis antigénica

La cantidad de antígeno es un factor que influye en la potencia y naturaleza de la respuesta inmunitaria inducida por una vacuna, determinando el tipo de respuesta, humoral o celular y el patrón de citoquinas (Hosken et al., 1995). La influencia de este factor se ha estudiado de forma extensa en la leishmaniosis. Sin embargo, los diferentes ensayos de vacunación arrojan datos controvertidos. Algunos estudios señalan el desarrollo de una respuesta de tipo Th1 tras la inmunización con dosis bajas (Ferrua et al., 2006; Kaur et al., 2008). Esta idea ha prevalecido durante mucho tiempo ya que en estudios de infección *in vivo*, los ratones infectados con dosis bajas de *Leishmania* fueron capaces de limitar la infección y desarrollar una respuesta inmunitaria de larga duración, generalmente asociada a un tipo Th1 (Bretscher et al., 1992; Doherty & Coffman, 1996; Menon & Bretscher, 1998). Sin embargo, otros trabajos señalan el desarrollo de una respuesta tipo Th2 cuando se inoculan dosis bajas de este parásito (Uzonna et al., 2004).

En la neosporosis, la información relativa a la influencia de la dosis antigénica sobre la respuesta inducida es escasa. Sólo un estudio de vacunación compara el efecto de la inoculación con dos dosis diferentes de parásito vivo (10^4 y 10^6) en un modelo murino cerebral, demostrándose protección en ambos grupos (Lunden et al., 2002). Dicha protección estuvo asociada en el grupo de dosis alta a niveles similares de IgG1 e IgG2a, y en los animales inoculados con la dosis más baja, a mayores niveles de IgG2a.

2.2.1.2. Uso de adyuvantes e inmunoestimuladores

Los adyuvantes son compuestos que se añaden a los preparados de vacunas con el fin de intensificar o modular la respuesta inmunitaria del organismo frente al antígeno administrado. La elección de un adyuvante adecuado se debe realizar teniendo en cuenta

la naturaleza del antígeno así como la vía de administración. Los adyuvantes determinan la magnitud, el tipo y la duración de la respuesta inmunitaria efectora mediante mecanismos que incluyen la presentación de antígeno y la distribución tisular, así como la fagocitosis y la digestión enzimática de la molécula (Heldens et al., 2008). Los diferentes mecanismos de acción de estas sustancias han sido extensamente revisados (Aucouturier et al., 2001; Schijns, 2001; Degen et al., 2003; Schijns, 2003; Singh & O'Hagan, 2003), sin embargo los acontecimientos exactos que inducen esta modificación en la respuesta inmunitaria no se conocen completamente.

Los adyuvantes pueden clasificarse según sus mecanismos de acción en tres grupos. Un primer grupo integrado por aquellos que tienen capacidad para formar un depósito o reservorio que permite la liberación lenta del antígeno, protegiéndolo de una degradación rápida y proporcionando un estímulo inmunogénico prolongado. Un segundo grupo compuesto por partículas fácilmente fagocitables, que incorporan el antígeno actuando como vehículo, permitiendo que las células presentadoras de antígeno lo atrapen y procesen más eficientemente que al antígeno libre. El tercer grupo de adyuvantes lo forman aquellos con capacidad de inmunomodulación, induciendo la síntesis de citoquinas por parte del animal vacunado. Además, se pueden obtener adyuvantes más potentes a partir de las combinación de los adyuvantes mencionados anteriormente, un ejemplo es el adyuvante completo de Freund formado por una emulsión oleosa, que actúa como depósito, y bacterinas de *Mycobacterium tuberculosis*, que se comportan como activadores de los macrófagos y células dendríticas. Los adyuvantes que con más frecuencia se han utilizado en la elaboración de vacunas con fines veterinarios son las sales de aluminio y emulsiones oleosas, que se incluyen en el grupo de adyuvantes que actúan como reservorio de antígeno.

La influencia de los adyuvantes en la protección inducida por vacunas inactivadas o de nueva generación frente a protozoos apicomplejos es bien conocida (McLeod et al., 1985; Daly & Long, 1996; Rafi-Janajreh et al., 2002). Aunque son escasos los estudios que comparan la eficacia protectora entre diferentes adyuvantes frente a la infección por *N. caninum*, muchos ensayos de vacunación han mostrado que la capacidad protectora de las formulaciones elaboradas a partir de antígeno inactivado depende fundamentalmente del adyuvante con el que se combine (Tabla 1). Así, Baszler et al. (2000) señalaron que la vacunación con antígeno soluble utilizando adyuvante completo de Freund o vesículas surfactantes no-iónicas exacerbó los signos clínicos nerviosos y originó una inadecuada respuesta inmunitaria predominantemente de tipo Th2. Sin embargo, la inmunización de ratones BALB/c con antígeno incluido en ISCOMS o mezclado con Quil A redujo la frecuencia de lesiones en el cerebro (Lunden et al., 2002). De manera similar, la vacunación con extracto soluble de taquizoítos combinado con RIBI® o con CpG redujo la presencia de parásito, las cargas de éste y las lesiones en el cerebro de ratones C57BL/6 (Cannas et al., 2003b; Ribeiro et al., 2009). En general, la protección observada en los anteriores estudios estuvo asociada a un predominio de una respuesta Th1 con producción de IFN-γ (Lunden et al., 2002; Ribeiro et al., 2009). En cuanto a la protección inducida frente a la neosporosis congénita, Liddell et al. (1999) vacunaron ratones hembra BALB/c con extracto completo de taquizoítos mezclado con el adyuvante ImmuMZXSR™ (Zona-

gen Inc.,TX, USA) antes de la cubrición, obteniéndose una protección eficaz frente a la transmisión vertical. Estos resultados contrastan con los obtenidos por Miller et al. (2005) quienes observaron que la vacunación con extracto soluble del aislado Nc-Nowra combinado con los adyuvantes VSA-3 o QuilA no previno la transmisión vertical en ratones. En lo que se refiere a las vacunas basadas en proteínas recombinantes, se han empleado mayoritariamente adyuvantes oleosos como los comerciales RIBI® o Titermax® Gold para llevar a cabo inmunizaciones con las proteínas recombinantes NcSAG1, NcSRS2, NcMIC3 (Cannas et al., 2003a; Cannas et al., 2003b), NcMIC1 (Alaeddine et al., 2005), NcGRA7 y NcSAG4 (Aguado-Martínez et al., 2009), los adyuvantes completo e incompleto de Freund con la proteína NcROP2 (Debache et al., 2008) y VSA-3 con NcGRA1, NcGRA2, NcMIC10 y Ncp24B (Ellis et al., 2008). También se han empleado otros adyuvantes como saponinas (NcMIC1, NcMIC3, NcROP2; Debache et al., 2008; Debache et al., 2009), toxina colérica (NcROP2, NcPDI, NcMAG1; Debache et al., 2010), liposomas (NcGRA7 y NcAMA1; Nishikawa et al., 2009; Zhang et al., 2010) y CpG (NcGRA7; Jenkins et al., 2004). Por lo general, en todos estos estudios la aplicación de la proteína con el adyuvante ha mejorado los resultados obtenidos con el empleo de la proteína sola.

En el ganado bovino, se comparó la respuesta inmunitaria inducida por la vacunación con taquizoítos enteros inactivados en combinación con cuatro adyuvantes diferentes (Havlogen, Polygen, Montanide ISA 773 y una mezcla de Havlogen y Bay R-1005), detectándose una ligera respuesta inmunitaria de base celular con el adyuvante Polygen (Andrianarivo et al., 2000). Sin embargo, dicha respuesta no fue suficiente para conferir protección frente a la infección fetal tras el desafío en el día 91 de gestación. De manera similar, la inmunización con extracto soluble del aislado Nc-Nowra combinado con VSA-3 no protegió frente a la mortalidad fetal tras el desafío en el día 70 de gestación (Williams et al., 2007). En condiciones de campo, sin embargo, la vacunación con Bovilis® Neoguard, vacuna a base de taquizoítos inactivados y adyuvante Havlogen, indujo una protección parcial frente al aborto (46%) en vacas infectadas naturalmente (Romero et al., 2004).

El papel de nuevos adyuvantes e inmunoestimuladores que resulten más seguros y eficaces frente a la infección por *N. caninum*, como la nanoencapsulación de proteínas que permitirían la liberación lenta de éstas y favorecerían una respuesta inmunitaria de tipo celular, se está investigando en la actualidad (Debache et al., 2011; Jiménez-Ruiz et al., 2011a).

Tabla 1. Tipos de adyuvantes utilizados en la vacunación frente a la neosporosis

Tipo de adyuvante		Th1	Th2	Linfocitos B	Linfocitos T $CD8^+$	Componentes	Criterio protección	Protección	Referencia
Reservorio	Emulsiones	++	-	+++	-	RIBI + prot. recombinantes	PCR cerebro	0-75%	Cannas et al., 2003b Alaeddine et al., 2005 Srinivasan et al., 2007
						RIBI + NSA	PCR cerebro	100%	Cannas et al., 2003b
						VSA-3 + prot. recombinantes/NSA	PCR crías	5-20%	Miller et al., 2005 Ellis et al., 2008
						Completo de Freund + NSA	Lesiones en cerebro	Exacerbación	Liddell et al., 1999
						ImmuMAXSR™ + NSA	PCR crías	100%	Baszler et al., 2000
Partículas que actúan de vehículo	Liposomas	+++	-	+	++	Liposomas + prot. recombinantes	PCR crías	55,6%	Zhang et al., 2010
						Liposomas + prot. recombinantes	% superv. crías	68,6%	Nishikawa et al., 2009
	ISCOMs	++	++	+++	+++	ISCOM + NSA	PCR cerebro	50%	Lunden et al., 2002
						ISCOM + prot. recombinantes	PCR cerebro	30-100%	Pinitkiatisakul et al., 2007
	Copolímeros no-iónicos	+	-	-	-	Vesículas no-iónicas surfactantes + NSA	Lesiones en cerebro	Exacerbación	Baszler et al., 2000
Inmuno-estimulantes	Saponinas	+++	++	+++	++	QuilA + NSA	PCR en cerebro	33%	Lunden et al., 2002
						Saponina + prot. recombinantes	Morbilidad % superv. crías Morbilidad	100% 25-50% 20-70%	Debache et al., 2008 Debache et al., 2009 Debache et al., 2010
	Ligando de TLR	+++	+	+	+	CpG + NSA	Cargas en cerebro	Protección	Ribeiro et al., 2009
						CpG + ESA		Exacerbación	
						CpG + vacuna-ADN	PCR crías	83%	Jenkins et al., 2004

2.2.1.3. Vía de administración

El lugar del organismo en el que se deposita la vacuna va a determinar la perfusión de ésta a través de circulación sanguínea o linfática, alcanzando los órganos linfoides secundarios y, por tanto, determinando la respuesta inmunitaria que se desarrollará tras la inmunización (Johansen et al., 2010). En otras protozoozis como la leishmaniosis, el papel de la vía de administración se ha demostrado en diversos experimentos. La vacunación con parásitos vivos por vía subcutánea favoreció un mayor reclutamiento de células inmunitarias en el sitio secundario de infección y la eliminación del parásito de manera más eficaz que la vía intradérmica (Tabbara et al., 2005). A su vez, la vía intramuscular ha sido la vía de elección para las vacunas de ADN. Sin embargo, la vía intradérmica ha demostrado ser más eficiente en el desarrollo de una inmunidad de larga duración que las vías intramuscular y subcutánea, permitiendo una disminución de la dosis antigénica (Méndez et al., 2002).

En la neosporosis, en cambio, la mayoría de los ensayos de vacunación han empleado las vías intraperitoneal y subcutánea, pero los efectos de este factor sobre el nivel y tipo de respuesta inmunitaria generada y el consecuente impacto sobre la infección han sido poco estudiados. La últimas investigaciones en este campo están probando nuevas vías como la inoculación intra-nasal de proteínas recombinantes de *N. caninum*, con la que se han obtenido mejores resultados de protección frente a la neosposis cerebral en el ratón con respecto a la vía clásica intraperitoneal (Debache et al., 2009; Debache et al., 2011).

2.2.2. Modelos animales para la evaluación de una vacuna

Durante el diseño de una vacuna, los ensayos preliminares para la evaluación de la eficacia y seguridad de las diferentes formulaciones candidatas se realizan habitualmente en animales de laboratorio. Estos modelos animales son una herramienta útil para realizar un primer cribado, rápido y económico, de las formulaciones diseñadas. En la neosporosis bovina, la vaca gestante constituye el animal de destino de estas formulaciones. Sin embargo, las exigencias que impone la experimentación con estos animales (elevado coste, tiempo empleado en la evaluación clínica, recogida de muestras y la dificultad de realizar un estudio exhaustivo de la infección que conllevaría un laborioso manejo de los animales y un alto número de sacrificios), hace inviable su utilización en las pruebas iniciales para elegir los mejores candidatos para desarrollar una vacuna.

La elección de un modelo para el ensayo de diferentes formulaciones va a depender fundamentalmente del objetivo que se busca con la vacunación: protección frente a la infección cerebral, transmisión transplacentaria o reactivaciones de la infección. El modelo murino ha demostrado ser una herramienta muy valiosa. En este sentido, se ha conseguido caracterizar ampliamente tanto la fase crónica de la infección, determinada por la presencia del parásito en el cerebro (neosporosis cerebral) (Lindsay et al., 1995; Long et al., 1998; Miller et al., 2002; Collantes-Fernández et al., 2006b), como la neosporosis congénita (Long & Baszler, 1996; Liddell et al., 1999; Quinn et al., 2002; López-Pérez et al., 2006; López-Pérez et al., 2008) y la respuesta inmunitaria inducida en el hospedador

(Khan et al., 1997; Ritter et al., 2002). No obstante, la comparación entre los modelos murinos es difícil debido a las diferentes variables empleadas en cada uno de los estudios, siendo las consecuencias de la neosporosis muy variables dependiendo de la estirpe de ratón, del aislado del parásito, dosis y vía de inoculación.

Diferentes estirpes consanguíneas, como la estirpe BALB/c, que presentan una elevada susceptibilidad a la infección dependiendo de la dosis y del aislado utilizado (Long et al., 1998; Collantes-Fernández et al., 2004), se han empleado eficazmente para evaluar el papel de la vacunación frente a la neosporosis cerebral (Baszler et al., 2000; Lunden et al., 2002; Bartley et al., 2008). El desafío con dosis subletales en estas estirpes consanguíneas surge como alternativa a modelos más agresivos que originan alta mortalidad en la fase aguda de la infección, permitiendo por un lado, la valoración de la eficacia de una vacuna frente a la infección crónica, diana de estudio de la infección cerebral, mediante la cuantificación de las lesiones o de la carga parasitaria en cerebro, y por otro, el empleo de modelos aceptables en materia de bienestar animal (Collantes-Fernández et al., 2006b; Ramamoorthy et al., 2007a). En la estirpe consanguínea BALB/c cuando se empleó una dosis moderada de inoculación de los aislados Nc-1 y Nc-Liverpool (10^6 taquizoítos) se observó que la infección presentaba tres fases. En la primera fase de la infección aguda (hasta día 7 post infección - PI.-), el parásito se diseminó orgánicamente, detectándose en sangre y en otros órganos como bazo, hígado y pulmón. A lo largo de la segunda semana PI (hasta día 13-14 PI) el desarrollo de la respuesta inmunitaria del ratón favoreció la eliminación del parásito de diversos órganos disminuyendo la frecuencia de detección y la carga parasitaria en estos órganos e incrementándose en el SNC. En la fase crónica de la infección (a partir del día 13-14 PI), *N. caninum* desapareció del pulmón y otros órganos y sólo se detectó en el SNC, donde inicialmente alcanzó una carga máxima (fase crónica temprana) para luego ir disminuyendo con el tiempo (fase crónica tardía) (Collantes-Fernández et al., 2006b).

Con respecto a los modelos de infección congénita, el modelo murino gestante ha mostrado ser un modelo óptimo para reproducir la transmisión transplacentaria de *N. caninum*, de manera que, como ocurre en el ganado bovino, el día de gestación en que tiene lugar la infección o el aislado con el que ésta se produce son factores que determinan tanto la mortalidad fetal como la transmisión vertical en el ratón (Long & Baszler, 1996; Liddell et al., 1999; López-Pérez et al., 2006; Ramamoorthy et al., 2007a; López-Pérez et al., 2008; Regidor-Cerrillo et al., 2010). En este sentido, el grupo SALUVET ha estandarizado un modelo de infección congénita en el que la infección con taquizoítos de un aislado virulento de *N. caninum* durante el segundo tercio (día 7-10 de gestación) en hembras gestantes de la estirpe BALB/c provoca una transmisión transplacentaria y una mortalidad neonatal (hasta el día 30 post parto) de más del 90%, significativamente mayores con respecto a la infección en los tercios primero y último de la gestación (López-Pérez et al., 2006; López-Pérez et al., 2008). Este modelo es altamente eficaz para el ensayo de formulaciones frente a la neosporosis congénita (Aguado-Martínez et al., 2009; Marugán-Hernández et al., 2011b). Sin embargo, dicho modelo se centra en el estudio de la infección congénita adquirida de manera exógena (TTEx). Recientemente, se ha estandarizado un modelo murino gestante en el que se evalúa la recrudescencia de la infección

en hembras crónicamente infectadas, mediante el estudio de la transmisión de la infección a su descencencia a lo largo de tres generaciones, con el objetivo de emular la TTEn descrita en el ganado bovino (Jiménez-Ruiz et al., 2011b). Dicho modelo puede resultar de gran utilidad para la evaluación de formulaciones vacunales dirigidas a proteger frente a los estadios del parásito que participan en la reactivación de la infección.

A pesar de la utilidad del uso del modelo murino como estrategia de cribado de diferentes formulaciones de vacunas, la evaluación de un candidato potencialmente válido ha de realizarse, en última instancia, en la especie de destino. La infección por *N. caninum* y sus principales consecuencias, el aborto y la transmisión vertical, se ha reproducido experimentalmente en numerosos estudios. A la hora de elegir un modelo que reproduzca la infección por *N. caninum* de manera similar a como ocurre en condiciones naturales en el ganado bovino, se han de tener en cuenta los diferentes factores que pueden influenciar las consecuencias de ésta y que se han explicado con anterioridad (apartado *1.5. Patogenia*). En este sentido, se ha conseguido inducir la mortalidad fetal asociada a la infección por *N. caninum* en un modelo bovino gestante, de manera altamente eficaz y reproducible, tras la inoculación por vía iv de un elevado número de taquizoítos (10^7-5×10^8) en el día 70 de gestación con los aislados Nc-1 o Nc-Liverpool (Williams et al., 2000; Williams et al., 2003; Macaldowie et al., 2004). La utilización de este modelo, se presenta como una herramienta útil para evaluar la seguridad y la eficacia de diferentes formulaciones de vacunas para prevenir el aborto mediantes ensayos de inmunización y desafío, como ya mostraron Williams et al. (2007).

2.2.3. Aproximaciones experimentales en el desarrollo de vacunas

En la actualidad, el desarrollo de vacunas frente a la neosporosis es un campo en el que se está trabajando intensamente. El mayor problema es la comparación objetiva entre los diferentes estudios ya que existe una gran variedad de modelos de infección empleados, aislados y dosis del desafío, vías de administración, especie animal y estirpe utilizada, adyuvantes y tipo de parámetros evaluados. En general, los resultados de protección más notables se han encontrado en los estudios de vacunación con aislados vivos atenuados de forma natural o artificial. Igualmente, se han ensayado vacunas inactivadas empleando taquizoítos inactivados o extractos antigénicos más o menos completos de los taquizoítos de *N. caninum*. La mayoría de estos estudios han arrojado resultados negativos o ambiguos en cuanto a protección frente a la neosporosis. Por otro lado, muchos investigadores se han centrado en el desarrollo de vacunas de nueva generación, como las basadas en diversas proteínas recombinantes. Aunque estos preparados han mostrado ser seguros, la respuesta inmunitaria que producen resulta ser insuficiente para proteger frente a la infección por *N. caninum*.

Independientemente de la tecnología aplicada para su obtención, el desarrollo de una vacuna frente a la neosporosis debería estar enfocado a la obtención de preparados que cumplan con los siguientes requisitos:

i. Seguridad, de especial importancia en vacunas vivas.

ii. Eficacia para proteger frente al aborto y reducir significativamente la transmisión vertical.

iii. Estabilidad, de forma que conserven sus propiedades desde la producción hasta su aplicación en campo, además de permitir su producción a gran escala para poder llevar a cabo su comercialización.

iv. Posibilidad de marcado, para discriminar animales vacunados de infectados de forma natural mediante la aplicación de técnicas de diagnóstico serológico.

2.2.3.1. Vacunas inactivadas

Las vacunas inactivadas presentan como ventaja, en comparación con las formulaciones vivas, el bajo riesgo de reversión a la virulencia. No obstante, la respuesta inmunitaria que inducen suele ser menos intensa y duradera, teniendo que administrar varias dosis de recuerdo para conseguir una inmunización completa y coadministrar adyuvantes que potencien de manera inespecífica la respuesta inmunitaria (Babiuk, 2002).

En el caso de las formulaciones con parásitos enteros, la inactivación de estos se ha conseguido tradicionalmente mediante métodos físicos o químicos. Sin embargo, la selección del método de inactivación plantea problemas ya que resulta difícil inactivar un microorganismo manteniendo intacta su completa antigenicidad (Babiuk, 2002). La inactivación física por calor se ha usado para protozoos como *Leishmania* (Rhee et al., 2002; Nagill et al., 2009), *T. gondii* (Haque et al., 1999) o *Plasmodium* (Hafalla et al., 2006). La desventaja de este método es que, en la mayoría de los casos, se somete al antígeno a elevadas temperaturas que pueden llevar a la desnaturalización de las proteínas, modificando sus propiedades antigénicas. Los métodos químicos que basan su actividad en la capacidad para modificar el material genético del microorganismo se utilizan habitualmente, sobre todo en vacunas frente a enfermedades víricas (Bahnemann, 1975), no obstante resultan potencialmente peligrosos durante el proceso de elaboración. El formaldehído se ha utilizado como método de inactivación en diversos estudios de vacunación frente a protozoos como *Leishmania major* (Holbrook & Cook, 1983; Mutiso et al., 2010) o *Tritrichomonas foetus* (Cobo et al., 2002; Cobo et al., 2004). Sin embargo, se han descrito problemas de este compuesto asociados a la reversión a la virulencia en vacunas de fiebre aftosa, junto con la descripción de importantes modificaciones en epítopos del antígeno vírico, lo que ha favorecido el uso de otros agentes alquilantes más seguros como los derivados de las aziridinas (etileneimina primaria, binaria o terciaria y *N*-acetiletileneimina) que actúan inactivando los virus por reacción con los ácidos nucléicos (Bahnemann, 1975; Burrage et al., 2000). Dentro de éstos, la etilenimina binaria (BEI), se ha utilizado ampliamente por ser más estable y menos tóxica que otras etileniminas (Bahnemann, 1975). La BEI actúa sobre el ácido nucléico sin dañar la estructura antigénica por lo que parece no alterar los epítopos del antígeno (Blackburn & Besselaar, 1991), logrando una mejor inducción de la inmunidad. Este agente se ha empleado para la inactivación de taquizoítos en la única vacuna que hasta la fecha se ha comercializado frente a la neosporosis bovina (Andrianarivo et al., 2000).

Por otro lado, las fracciones inmunizantes naturales del parásito, como diferentes extractos (soluble, insoluble o total) surgen como candidatos potenciales debido a que incluyen un completo abanico de antígenos, y su utilización es más segura que la de las vacunas inactivadas de organismos enteros. La mayoría de las vacunas inactivadas que se han ensayado frente a la neosporosis emplean como antígeno un extracto soluble de taquizoítos, obtenido tras la lisis del parásito mediante sonicación y ciclos de calor y frío, centrifugación y eliminación del detritus celular (Liddell et al., 1999; Baszler et al., 2000; Lunden et al., 2002; Cannas et al., 2003b; Miller et al., 2005; Williams et al., 2007; Ribeiro et al., 2009). Dicho extracto contiene proteínas citoplasmáticas y proteínas de micronemas, roptrias y gránulos densos, implicadas en procesos de adhesión, invasión, formación de la vacuola parasitófora y regulación de los procesos de interacción célula-parásito. Sin embargo, carece de proteínas de superficie, altamente inmunogénicas e implicadas en procesos de adhesión del parásito a la célula hospedadora (Hemphill et al., 2006). Los resultados de protección obtenidos tras la inmunización con estas fracciones antigénicas son muy variables y dependen fundamentalmente del adyuvante utilizado, como se ha explicado anteriormente (Tablas 2a y 2b). Por último, el uso de fracciones enriquecidas en proteínas de organelas como micronemas o roptrias, de gran importancia durante la invasión celular del parásito como se ha visto en *T. gondii* (Dubremetz, 2007), está cobrando interés en el desarrollo de vacunas frente a la neosporosis (Marugán-Hernández et al., 2011c). Recientemente se ha evaluado en un modelo murino la protección inducida por una fracción rica en proteínas excretadas y secretadas de *N. caninum* (ESA) frente a la infección (Ribeiro et al., 2009). Estas proteínas indujeron una intensa respuesta celular que estuvo asociada a una mayor susceptibilidad a la infección.

Tabla 2a. Vacunas inactivadas ensayadas en modelos murinos frente a la neosporosis cerebral y congénita.

Modelo	Inmunización (tipo de antígeno, aislado y dosis)	Adyuvante	Criterio protección[a]	Protección[b]	Respuesta inmunitaria	Referencia
Murino de infección cerebral	Inactivación por frío (mutante Ncts-8) 2×10^6 s.c.	-	PCR cerebro	14,3%	-	Lindsay et al., 1999b
	NSA (Nc-1) 2 µg-8 µg s.c.	ISCOM	PCR cerebro	50%	Mixta Th1/Th2	Lunden et al., 2002
		QuilA		33%	Mixta Th1/Th2	
		-		14%	Mixta Th1/Th2	
	NSA (Nc-1) 50 µg s.c.	Vesiculas no-iónicas surfactantes	Frecuencia de lesiones en cerebro	Exacerbación 11,2±2,7 *vs* 1,8±1,3	Predominio Th2	Baszler et al., 2000
	-			No protección 0,7±1,2 *vs* 1,8±1,3	Predominio Th1	
	NSA (Nc-1) 50 µg s.c.	Completo de Freund		Exacerbación 4,71±1,68 *vs* 1,8±1,3	Predominio Th2	
	-			No protección 1±1,5 *vs* 1,8±1,3	-	
	NSA (Nc-1) 250 µg/ml i.p.	RIBI®	PCR cerebro	100%	-	Cannas et al., 2003b
	-			43%	-	
	NSA (Nc-1) 25 µg s.c.	CpG	Cargas cerebro	Protección ~1 taquiz/100 ng tejido	Predominio Th1	Ribeiro et al., 2009
		-		Protección ~1 taquiz/100 ng tejido	Predominio Th2	
	ESA (Nc-1) 25 µg s.c.	CpG		Exacerbación ~1500 taquiz/100 ng tejido	Predominio Th1	
		-		No protección ~550 taquiz/100 ng tejido	Predominio Th1	
Murino de infección congénita	Extracto total (Nc-1) 5 µg s.c.	ImmuMAXSR™ (emulsión)	PCR crías	100%	-	Liddell et al., 1999
	-			2%-28%	-	
	NSA (Nc-Nowra) 10 µg s.c.	VSA-3® (emulsión)	PCR crías	18,1%-20,9%	Predominio IgG1	Miller et al., 2005
	-			2,6%-4,5%	Baja inducción de anticuerpos	

[a]: El criterio elegido de entre todos los parámetros evaluados por su significación frente al control no vacunado desafiado

[b]: Protección calculada como porcentaje respecto al control no vacunado desafiado

NSA: antígeno soluble de *N. caninum*

ESA: proteínas excretadas-secretadas

Tabla 2b. Vacunas inactivadas ensayadas en modelos bovinos frente a la neosporosis.

Inmunización (tipo de antígeno, aislado y dosis)	Adyuvante	Desafío (aislado y dosis)	Criterio protección[a]	Protección[b]	Respuesta inmunitaria	Referencia
TZ enteros inactivados por BEI (Nc-BPA1) 2 ×400 µg s.c.	Polygen™ (copolímero)	Nc-BPA1 2 × (2×10^7) s.c. + i.m. 91 dg	Aborto Transmisión vertical	28,6% 0%	↑ inducción de anticuerpos ↑ respuesta celular	Andrianarivo et al., 2000
-				60% 0%	↓ inducción de anticuerpos ↑ respuesta celular	
TZ enteros inactivados por BEI (Nc-BPA1) 3 ×400 µg s.c	Polygen™ (copolímero)	Infección natural	Transmisión vertical	0%	↑ inducción de anticuerpos ↑ respuesta celular	Andrianarivo et al., 2005
TZ enteros inactivados (Neoguard®) 3 × 10^6/ml s.c.	Polygen™ (copolímero)	Estado desconocido respecto a la infección	Aborto	46,2%	-	Romero et al., 2004
Extracto total (Nc-Nowra) 2 ×100 µg s.c.	VSA-3	Nc-Liv 1 × 10^7 i.v. 70 dg	Aborto	0%	↑ inducción de anticuerpos ↓ respuesta celular	Williams et al., 2007
	QuilA			0%		
-	VSA-3 o QuilA			0%	↓ inducción de anticuerpos ↓ respuesta celular	
TZ enteros inactivados (Bovilis Neoguard®) 3 × 10^6/ml s.c.	Havlogen®	Infección natural	Aborto	25%	↑ inducción de anticuerpos	Weston et al., 2011

[a]: El criterio elegido de entre todos los parámetros evaluados por su significación frente al control no vacunado desafiado

[b]: Protección calculada como porcentaje respecto al control no vacunado desafiado

TZ: taquizoítos

2.2.3.2. Vacunas vivas

La principal característica de estas vacunas es que utilizan microorganismos vivos, que pueden replicarse en el hospedador sin causar enfermedad. El éxito de las vacunas vivas frente a infecciones por protozoos apicomplejos, como las comercializadas frente a la infección por *Eimeria* o *T. gondii,* radica en que estas formulaciones permiten un adecuado procesamiento y presentación del antígeno al sistema inmunitario que lo estimula apropiadamente y lo dirige hacia una respuesta mediada por células, proporcionando una memoria inmunológica de larga duración, simulando la inducida durante la infección natural (Innes & Vermeulen, 2006). Sin embargo, el principal problema que plantean es que los parásitos reviertan a la virulencia. En este sentido, se han llevado a cabo diferentes estrategias que aseguren el uso de parásitos cuya persistencia en el hospedador sea nula o muy baja, pero capaces de generar una respuesta inmunitaria protectora, atendiendo así a criterios de seguridad y eficacia. Entre ellas, los mecanismos más utilizados en la generacion de vacunas vivas frente las infecciones producidas por protozoos apicomplejos, incluido *N. caninum*, han sido la atenuación por pases sucesivos en cultivos celulares, la utilización de métodos físico-químicos y la obtención de parásitos atenuados naturalmente (Tabla 3).

La atenuación mediante pases sucesivos en cultivos celulares se basa en la adaptación del parásito a crecer en condiciones inusuales, de manera que pierda la capacidad de adaptación a su hospedador habitual. Esta técnica se ha utilizado exitosamente para la obtención de la única vacuna comercial frente a la toxoplasmosis ovina, Ovilis Toxovax® (Intervet-Schering Plough). Dicha vacuna está elaborada a partir de un aislado procedente de un feto ovino, que carecía de la capacidad de formar quistes tisulares y que fue atenuado mediante sucesivos pases (× 3000) en ratón (Wilkins et al., 1988), demostrando posteriormente la capacidad de inducir una respuesta protectora frente al aborto (Buxton et al., 1991; Buxton et al., 1993). En la neosporosis, el pase seriado en cultivo del aislado Nc-1 (× 88) redujo su virulencia cuando se comparó con el aislado original (× 43) (Bartley et al., 2006) y fue capaz de reducir parcialmente la infección cerebral en un modelo murino (Bartley et al., 2008).

La atenuación por métodos físico-químicos, como la irradiación con rayosγ, que bloquea la capacidad de replicación del parásito pero no su capacidad invasiva, ha sido probada en *T. gondii* (Hiramoto et al., 2002), en *Plasmodium* (Chatterjee et al., 2001; Silvie et al., 2002) y en *Eimeria* (Jenkins et al., 1997). Un factor limitante de este método es la dosis irradiada. La sobredosificación puede inactivar al parásito y una dosis excesivamente baja puede permitir que el parásito continúe su desarrollo normal (Nussenzweig et al., 1967). En esta línea, se ha obtenido un aislado de *N. caninum* irradiado con rayos γ a partir del aislado original Nc-1 (Ramamoorthy et al., 2006). La inmunización con taquizoítos atenuados de dicho aislado previno la mortalidad en ratones infectados con dosis letales de un aislado virulento (Ramamoorthy et al., 2006; Ramamoorthy et al., 2007b). La atenuación por mutagénesis química y posterior selección de mutantes en función de su capacidad de multiplicarse a temperaturas inferiores a la del hospedador (mutantes sensibles a temperatura o ts), permite la obtención aislados con una capacidad de replicación menor que las variantes originales, con lo que serán menos virulentos sin perder su

inmunogenicidad. El aislado ts-4 de *T. gondii* es un mutante termo-sensible que ha sido ampliamente utilizado en ensayos de inmunización frente a aislados virulentos de *T. gondii* en ratón por su incapacidad de formar quistes tisulares y su baja persistencia en el hospedador (Pfefferkorn & Pfefferkorn, 1976; Waldeland et al., 1983). Dicho aislado es capaz de conferir protección frente a la toxoplasmosis aguda y congénita y de reducir la formación de quistes tisulares (Waldeland & Frenkel, 1983; McLeod et al., 1988; Gazzinelli et al., 1991). Esta metodología tambien se ha aplicado a la obtención de aislados atenuados de *N. caninum*. El mutante termosensible Ncts-8, redujo la frecuencia de presentación de lesiones en encéfalo así como la gravedad de éstas (Lindsay et al., 1999b).

El uso de parásitos atenuados naturalmente, obtenidos de animales asintomáticos, se ha utilizado en la coccidiosis aviar, existiendo en el mercado vacunas que contienen cepas de *Eimeria* que presentan baja patogenicidad de forma natural y que protegen frente a la enfermedad (Vermeulen et al., 2001). Los primeros trabajos de vacunación frente a la neosporosis siguieron esta aproximación. La inmunización con taquizoítos vivos del aislado de *N. caninum* de virulencia reducida Nc-SweB1 fue capaz de inducir una respuesta protectora, disminuyendo significativamente la mortalidad en ratones (Atkinson et al., 1999). A pesar de la protección inducida, la vacunación en ratones con el aislado Nc-SweB1, produjo signos clínicos en el 20% de los animales vacunados, demostrando ser un aislado poco seguro (Atkinson et al., 1999). Otros aislados como el JPA1 y el NcNowra, obtenidos a partir de animales infectados pero clínicamente sanos, mostraron una virulencia reducida de forma natural cuando se caracterizaron en un modelo murino. Dichos aislados provocaron alteraciones más leves que el aislado Nc-Liverpool en los diversos órganos estudiados (Shibahara et al., 1999; Miller et al., 2002). Esto sugiere la idea de que aquellos aislados obtenidos a partir de animales sanos podrían mostrar una menor virulencia. En la actualidad, las pruebas de protección utilizando aislados de *N. caninum* naturalmente atenuados han mostrado resultados muy esperanzadores. La inmunización con taquizoítos del aislado australiano naturalmente atenuado Nc-Nowra (Miller et al., 2002) ha demostrado proteger frente a la transmisión vertical en ratones gestantes (Miller et al., 2005) y frente a la mortalidad fetal en un modelo bovino gestante (Williams et al., 2007). A pesar de estos resultados, hasta el momento, el número de aislados referenciados en la bibliografía procedentes de animales sanos pero congénitamente infectados es muy reducido, debido principalmente a que el proceso de aislamiento de *N. caninum* a partir de tejidos infectados es muy laborioso y difícil. En este sentido, en los últimos años, el grupo SALUVET ha estandarizado una técnica de aislamiento en cultivo, utilizando como paso previo la amplificación en ratones atímicos desnudos (Regidor-Cerrillo et al., 2008). Fruto de este trabajo, se ha obtenido una batería de aislados de origen español a partir del cerebro de terneros sanos pero congénitamente infectados que han sido mantenidos en cultivos celulares en un número de pases controlado (Regidor-Cerrillo et al., 2008). Esto supone la disponibilidad de un número elevado de aislados de *N. caninum* con sus características biológicas inalteradas como consecuencia de un mantenimiento prolongado en cultivo (Bartley et al., 2006). Dichos aislados constituyen una ventajosa herramienta para el desarrollo de nuevos productos sanitarios de utilidad inmunoprofiláctica frente a la neosporosis.

2.2.3.3. Vacunas de nueva generación

Las vacunas de nueva generación basadas en proteínas recombinantes o en la inoculación de ADN o vectores vivos que codifican determinadas proteínas del parásito, han sido también probadas para el control de la neosporosis. Las proteínas diana para el diseño de estas vacunas son aquellas implicadas en procesos de adhesión, invasión y proliferación del parásito, como diversas proteínas inmunodominantes de la superficie del taquizoíto o de organelas de secreción (gránulos densos, micronemas y roptrias) (Innes & Vermeulen, 2006). Este grupo de vacunas tienen las ventajas de la inocuidad y de la fácil elaboración. Pero, desafortunadamente, inducen baja inmunidad debido principalmente a la corta vida media que presentan, y a la escasa activación de las células presentadoras de antígeno (Aichele et al., 1995). Por ello, el uso de adyuvantes que potencien y favorezcan una correcta presentación de estos antígenos a las células del sistema inmunitario es determinante para obtener resultados eficaces (Jenkins et al., 2004; Nishikawa et al., 2009; Debache et al., 2011).

En la infección por *N. caninum*, la proteína de superficie NcSRS2 mostró buenos resultados en lo que concierne a la protección frente a la transmisión transplacentaria en modelos murinos gestantes (Nishikawa et al., 2001b; Haldorson et al., 2005). La vacunación con diversas proteínas de micronemas y gránulos densos indujo una protección parcial con una reducción de la carga parasitaria en los animales vacunados y sus crías (Jenkins et al., 2004; Alaeddine et al., 2005; Cho et al., 2005; Ramamoorthy et al., 2007b). Recientemente se ha valorado la proteína de roptrias NcROP2 que, aunque no protegió frente a la transmisión vertical, sí que redujo la carga parasitaria en las crías (Debache et al., 2009). La combinación de varias proteínas a la vez también mostró mejores resultados de protección que la inmunización con cada proteína por separado (Cho et al., 2005; Debache et al., 2009).

Los avances en ingeniería genética han permitido la creación de un gran número de parásitos mutantes vivos atenuados, defectivos en genes que tienen funciones esenciales en la supervivencia el parásito en el hospedador. Las técnicas de genética reversa se han utilizado con éxito en malaria para conseguir parásitos genéticamente modificados, que han conferido elevada protección en numerosos estudios en modelos experimentales (Vaughan et al., 2010). El grupo SALUVET ha empleado por primera vez en *N. caninum* estas técnicas para la expresión homóloga y estable de la proteína específica del estadio de bradizoíto NcSAG4 en el estadio de taquizoíto, obteniéndose de esta forma dos nuevas cepas transgénicas denominadas Nc-1 SAG4c1.1 y Nc-1 SAG4c2.1 (Marugán-Hernández et al., 2011a), capaces de conferir protección frente a la neosporosis congénita al ser empleadas como vacunas vivas en un modelo murino (Marugán-Hernández et al., 2011b) (Tabla 3).

Tabla 3. Vacunas vivas ensayadas en modelos animales frente a la neosporosis cerebral y congénita.

Modelo	Inmunización (aislado y dosis)	Criterio protección[a]	Protección[b]	Respuesta inmunitaria	Referencia
Murino de infección cerebral	Nc-SweB1 (atenuado naturalmente) 1×10^6	Tasa de supervivivencia	62,5%	-	Atkinson et al., 1999
	Nc-1 (dosis subletal) 1×10^4 i.p.	Frecuencia de lesiones en cerebro	67%	Predominio Th1	Lunden et al., 2002
	Nc-1 (dosis subletal) 1×10^6 i.p.	Frecuencia de lesiones en cerebro	10%	Predominio Th1	
	Nc-1 (γ-irradiación) 1×10^6 i.p.	Tasa de supervivivencia	100%	Mixta Th1 / Th2	Ramamoorthy et al., 2006
		Frecuencia de lesiones en cerebro	50%	Mixta Th1 / Th2	
	Nc-1 (γ-irradiación) 1×10^6 i.p.	Gradación de lesiones en cerebro	96%	-	Ramamoorthy et al., 2007a
	Nc-1 (γ-irradiación) 1×10^6 i.p.	Tasa de supervivivencia	100%	Predominio Th1	Ramamoorthy et al., 2007b
	Nc1 p.88 (pases en cultivo) 1×10^6 i.p.	PCR en cerebro	44%	-	Bartley et al., 2008
	Nc1 p.43 (virulento) 1×10^6 i.p.	PCR en cerebro	14%	-	
Murino de infección congénita	Nc-Nowra (atenuado naturalmente) 1×10^4 s.c	PCR crías	89,5%-98,8%	Mixta Th1 / Th2	Miller et al., 2005
	Nc-1 (γ-irradiación) 1×10^6 i.p.	PCR crías	26,7%	Predominio Th1	Ramamoorthy et al., 2007
	Nc-1 SAG4[c] 1.1 (mutante transgénico) 5×10^5 s.c.	PCR crías	47,4%	Mixta Th1 / Th2	Marugan-Hernández et al., 2011b
	Nc-1 SAG4[c] 2.1 (mutante transgénico) 5×10^5 s.c.	PCR crías	11,7%	Mixta Th1 / Th2	
Bovino gestante	Nc-1 (sin atenuar) 1×10^7+1×10^8 s.c.	Transmisión	100%	↑ inducción de anticuerpos ↑ proliferación celular y producción IFN-γ	Innes et al., 2001
	Nc-Nowra (atenuado naturalmente) 1×10^7 i.v.	Aborto	100%		Williams et al., 2007

[a]: El criterio elegido de entre todos los parámetros evaluados por su significación frente al control no vacunado desafiado

[b]: Protección calculada como porcentaje respecto al control no vacunado desafiado

Por lo general, las vacunas de nueva generación han mostrado una menor eficacia, en lo que a protección se refiere, que las vacunas vivas. Sin embargo, junto con las vacunas vivas, se erigen como una de las mejores alternativas para el desarrollo de formulaciones frente a la infección por *N. caninum,* ya que muestran mayor seguridad, estabilidad, facilidad de producción a gran escala desde el punto de vista industrial e incluso posibilidad de marcado. Con respecto a este último punto, ninguna preparación ensayada hasta el momento permite diferenciar animales vacunados de infectados (Reichel & Ellis, 2009). En este sentido, cabe señalar que la inoculación de las cepas transgénicas Nc-1 SAG4c1.1 y Nc-1 SAG4c2.1 produjo una elevada producción de anticuerpos frente a la proteína NcSAG4 (Marugán-Hernández et al., 2011a; Marugán-Hernández et al., 2011b). Esta respuesta podría permitir diferenciar los ratones inmunizados con las cepas transgénicas de los no inmunizados. Si estas diferencias fueran reproducibles en modelos bovinos, el empleo de estas cepas transgénicas como vacuna podría ser útil para el reconocimiento de los animales vacunados.

3. Justificación y objetivos

N. caninum es un protozoo apicomplejo considerado como una de las principales causas de aborto en el ganado bovino a nivel mundial, habiéndose identificado hasta en un 60% de los fetos abortados enviados a laboratorios veterinarios de diagnóstico (Aduriz et al., 2000; Moore et al., 2003; Anderson, 2007). La magnitud de las pérdidas económicas asociadas a la neosporosis bovina pone de manifiesto la necesidad de establecer medidas eficaces para el control de la enfermedad. Entre las numerosas estrategias de control planteadas, la vacunación ha sido señalada como la más rentable económicamente (Reichel & Ellis, 2006; Reichel & Ellis, 2009). Sin embargo, hasta la fecha, sólo se ha comercializado una vacuna frente a la neosporosis basada en taquizoítos inactivados (Andrianarivo et al., 2000), mostrando una limitada eficacia en la prevención de abortos (inferior al 50%) como indican varios trabajos de campo (Romero et al., 2004; Weston et al., 2011). Ya que diferentes estudios basados en modelos económicos sugieren la vacunación como una alternativa viable para el control de la neosporosis bovina y, en la actualidad, no existe ninguna vacuna en el mercado, el desarrollo de una formulación eficaz y segura sería un importante avance en este campo de investigación.

La naturaleza intracelular de *N. caninum* sugiere un importante papel de la inmunidad mediada por células en el control de la infección (Innes et al., 2002; Hemphill et al., 2006). Una respuesta inmunitaria celular de tipo Th1, asociada a la producción de citoquinas como la IL-12 o el IFN-γ, parece ser crucial para la resistencia del hospedador frente a la infección (Baszler et al., 1999; Long & Baszler, 2000; Ritter et al., 2002). Sin embargo, este tipo de respuesta desarrollada durante la gestación podría comprometer la viabilidad fetal. Por el contrario, una respuesta de tipo Th2, que ayuda al mantenimiento de la gestación, podría favorecer la transmisión vertical del parásito (López-Pérez et al., 2008; López-Pérez et al., 2011). Estos datos parecen sugerir que una vacuna diseñada frente a un parásito como *N. caninum* debería inducir una respuesta inmunitaria adquirida de tipo Th1, capaz de controlar la infección por el parásito, sin comprometer la gestación.

Por otro lado, una vacuna frente a *N. caninum* debería diseñarse en función de los objetivos que se pretenden alcanzar, ya sea prevenir los signos clínicos de la enfermedad (aborto o transmisión vertical) o controlar e incluso erradicar la infección en el rebaño, interviniendo en diferentes puntos del ciclo biológico del parásito. Por ejemplo, una diana de la vacuna frente a *N. caninum* podría ser evitar la recrudescencia de una infección crónica y sus consecuencias. Para ello, la vacuna debería estar dirigida a evitar los procesos de tranformación de bradizoíto a taquizoíto o a limitar la parasitemia resultante de una reactivación, protegiendo así frente a la consiguiente transmisión transplacetaria. Por otro lado, una vacunación encaminada a evitar primoinfecciones relacionadas con los brotes de abortos de tipo epidémico debería dirigirse a prevenir la infección que acontece tras la ingestión de ooquistes.

En los últimos años, se han llevado a cabo diferentes estrategias para el desarrollo de vacunas frente a la infección por *N. caninum* que incluyen vacunas inactivadas, vivas y de nueva generación basadas en la tecnología del ADN recombinante (Innes et al., 2011). A pesar de que las vacunas de nueva generación se presentan como una alternativa intere-

sante para el futuro, en principio, aquellas formulaciones que contienen sólo algunos antígenos del parásito podrían inducir una respuesta inmunitaria menos eficaz que las que contienen antígenos del organismo completo. De hecho, hasta la fecha, la mayoría de las vacunas veterinarias disponibles en el mercado son vacunas a partir de organismos vivos o enteros inactivados, obtenidos mediante tecnología convencional (Meeusen et al., 2007). En general, las vacunas inactivadas han resultado ser menos efectivas que las vacunas vivas, sin embargo, son formulaciones más estables y no presentan el riesgo de reversión a la virulencia. Por el contrario, en términos de inmunoprotección frente a las infecciones por protozoos, las vacunas vivas son capaces de inducir una respuesta inmunitaria mucho más parecida a la que tiene lugar durante las infecciones naturales (Innes et al., 2011). Las desventajas de este tipo de vacunas tienen que ver con el elevado coste de producción, la corta vida de la formulación, los problemas de conservación y la posibilidad de reversión a la virulencia. El uso de organismos naturalmente atenuados como vacuna viva frente a la neosporosis está bajo estudio, habiendo mostrado resultados de protección prometedores, tanto en modelos de laboratorio como en el ganado bovino (Miller et al., 2005; Williams et al., 2007). Varios estudios evidencian que algunos aislados procedentes de animales con infección asintomática podrían presentar una reducida virulencia, siendo por tanto razonables candidatos para la elaboración de una vacuna viva frente a esta enfermedad (Miller et al., 2002; Pereira García-Melo et al., 2010; Regidor-Cerrillo et al., 2010), dada su teórica seguridad, requisito de suma importancia en estas vacunas.

En el desarrollo de vacunas frente a la neosporosis y, en general, frente a cualquier protozoo, juega un papel esencial la selección de los antígenos y de los sistemas de liberacion que permitan su correcto procesamiento y presentación al sistema immunitario del hospedador, permitiendo la generación de respuestas inmunitarias innata y adquirida eficaces (Innes & Vermeulen, 2006). Algunos factores relacionados con la composición antigénica, como la dosis o el tipo de preparado (parásito entero, extracto soluble, extracto completo, etc.), pueden determinar la presentación y reconocimiento antigénico, modulando el tipo de respuesta inmunitaria desarrollada (de tipo Th1 o Th2). Por otro lado, debido a la complejidad del ciclo biológico de *N. caninum,* que posiblemente implica la presentación de una gran diversidad de antígenos específicos de estadio, posiblemente sea necesario desarrollar formulaciones que contengan antígenos de diferentes estadios. Este enfoque podría ser beneficioso para interrumpir procesos parasitarios importantes como la conversión de bradizoíto a taquizoíto, relacionado con la persistencia del parásito en el hospedador, o como el ciclo lítico del taquizoíto durante la fase aguda de la enfermedad.

Una vez identificado la composición antigénica de una formulación, otro punto importante para el desarrollo de una vacuna es la selección de un adyuvante capaz de estimular una respuesta inmunitaria protectora de tipo Th1. En la neosporosis, se han utilizado diferentes adyuvantes, principalmente para maximizar la eficacia de vacunas que incluyen proteínas recombinantes o el parásito inactivado. Sin embargo, muy pocos autores comparan la eficacia de los adyuvantes (Andrianarivo et al., 2000; Baszler et al., 2000; Lunden et al., 2002) y los diferentes resultados observados tras la inmunización con estas formulaciones probablemente sean atribuibles al tipo de antígeno utilizado. Puesto que los adyuvantes juegan un papel crítico en el diseño de vacunas inactivadas, es

necesaria la realización de estudios que permitan comprender el papel de éstos en la protección frente a la neosporosis.

El objetivo de la presente Tesis Doctoral fue el desarrollo de vacunas frente a la neosporosis bovina a partir de parásitos inactivados y vivos obtenidos mediante tecnologías convencionales. Para ello, se probaron diversas composiciones antigénicas basadas, por un lado, en la mezcla de parásito entero inactivado combinado con diferentes adyuvantes y, por el otro, en parásito vivo obtenido a partir de aislados procedentes de animales asintomáticos que pudieran resultar seguros pero capaces de inducir una respuesta inmunitaria protectora en el hospedador. La seguridad y eficacia de las formulaciones se evaluaron en diferentes modelos animales de infección cerebral y congénita.

Con este propósito, se plantearon los siguientes objetivos.

Objetivo 1 (Capítulo II y III). Desarrollo de vacunas inactivadas frente a la neosporosis basadas en la utilización de zoítos de *N. caninum*.

Para la ejecución de esta parte, se propusieron los siguientes subobjetivos:

Subobjetivo 1.1. (Capítulo II). Influencia del adyuvante y la dosis antigénica en la protección conferida por una vacuna inactivada frente a la neosporosis cerebral en un modelo murino.

En este capítulo, se estudió el papel en la protección frente a la neosporosis de tres adyuvantes diferentes: emulsión de agua en aceite e hidróxido de aluminio en combinación con CpG-ODN o con extracto de Ginseng; y tres dosis de antígeno. Para probar la eficacia y seguridad de las diferentes formulaciones se utilizó una estrategia de ensayo-error. La eficacia, seguridad e inmunogenicidad de los preparados se determinaron tanto en la fase aguda como crónica de la enfermedad utilizando un modelo de neosporosis cerebral en ratones BALB/c.

Subobjetivo 1.2. (Capítulo III). Influencia de la inmunización con zoítos enteros inactivados procedentes de diferentes estadios del ciclo biológico de *N. caninum* sobre la protección frente a la neosporosis congénita y cerebral.

En este capítulo se investigó el papel de los antígenos procedentes de los estadios de taquizoíto y bradizoíto, así como el de diferentes adyuvantes en la prevención de la infección por *N. caninum*. Para ello, se ensayaron varias formulaciones que contenían taquizoítos enteros o una mezcla de taquizoítos y bradizoítos enteros, inactivados e incorporados en varios de los adyuvantes probados previamente. Para la inmunización se empleó un aislado de *N. caninum*, Nc-Spain7, con una elevada capacidad de transformación de taquizoíto a bradizoíto *in vitro*. La eficacia y seguridad de las formulaciones se evaluaron en sendos modelos murinos de infección cerebral y congénita. La seguridad se determinó diariamente mediante observación y palpación de los animales para la detección de reacciones adversas. La eficacia frente a la infección congenital se midió determinando las tasas de mortalidad neonatal y de transmisión vertical. El estudio de la eficacia de la

vacunación frente a la neosporosis cerebral se llevó a cabo mediante el análisis de la presencia del parásito en el cerebro de ratones adultos. Adicionalmente, la respuesta inmunitaria se determinó mediante analisis serológicos y de expresión de citoquinas antes y después del desafío.

Objetivo 2 (Capítulos IV, V y VI). Desarrollo de una vacuna viva frente a la neosporosis basada en la utilización de un aislado naturalmente atenuado de *N. caninum*.

Subobjetivo 2.1. (Capítulo IV). Aislamiento y caracterización de un aislado bovino de *N. caninum* para su uso como candidato en el desarrollo de vacunas frente a la neosporosis bovina.

En el capítulo IV se abordó la obtención y caracterización de un nuevo aislado de *N. caninum,* Nc-Spain 1H, a partir del cerebro de un ternero clínicamente sano pero congénitamente infectado. Con el objetivo de evaluar su virulencia y por tanto capacidad como candidato seguro para una vacuna viva, se estudió su comportamiento biológico tanto *in vitro* como *in vivo*. El rendimiento en la producción de taquizoítos, la capacidad de infección del aislado y la tasa de conversión de taquizoíto a bradizoíto se estudiaron mediante ensayos *in vitro*. Los estudios de patogenicidad se llevaron a cabo utilizando modelos de infección en la estirpe BALB/c. De esta manera, la capacidad de transmisión del aislado Nc-Spain 1H a la descendencia se valoró en un modelo murino gestante, mientras que la capacidad del aislado para producir la infección cerebral se evaluó en un modelo murino no gestante.

Subobjetivo 2.2. (Capítulo V). Caracterización de un aislado naturalmente atenuado en un modelo bovino gestante.

En este capítulo, se estudió la seguridad del aislado anterior tras su inoculación intravenosa a novillas en el día 70 de gestación. Para elló se determinó la capacidad del aislado de producir muerte fetal en el primer tercio de la gestación. Complementariamente, se evaluó su inmunogenicidad mediante la investigación de la respuesta inmunitaria humoral y celular desarrolladas.

Subobjetivo 2.3. (Capítulo VI). Evaluación de la protección conferida por un aislado naturalmente atenuado de *N. caninum* frente a la neosporosis congénita y cerebral.

En este apartado, se estudió la respuesta inmunoprotectora frente a la neosporosis congénita y cerebral inducida tras la inmunización con taquizoítos vivos del aislado Nc-Spain 1H en ratones BALB/c. La seguridad del aislado en estudio se determinó mediante el análisis de la presencia del parásito en el cerebro tras la inmunización. Para estudiar la protección frente a la infección congénita, se midieron las tasas de mortalidad neonatal y de transmisión vertical. El estudio de eficacia frente a la neosporosis cerebral se llevó a cabo mediante el análisis de la presencia del parásito en el cerebro de ratones adultos. También se midieron las respuestas inmunitarias celular y humoral inducidas tras la va-

cunación. Adicionalmente, se evaluó la influencia de la dosis de inmunización en la protección frente a las infecciones congénita y cerebral.

Justification and objectives

The apicomplexan protozoan *N. caninum* is considered one of the major causes of abortion in cattle worldwide, having been found in up to 60% of aborted fetuses submitted to veterinary diagnostic laboratories (Aduriz et al., 2000; Moore et al., 2003; Anderson, 2007). The magnitude of the industry economic losses as a result of bovine neosporosis underlines the need for efficient strategies to control the disease. Among the many control measures that have been proposed, vaccination has been highlighted as the most cost-effective approach (Reichel & Ellis, 2006; Reichel & Ellis, 2009). Nevertheless, so far, only a vaccine based on inactivated tachyzoites (Andrianarivo et al., 2000) has been marketed in different countries and its limited efficacy in preventing abortion (less than 50%) has been previously pointed out in field research (Romero et al., 2004; Weston et al., 2011). Thus, since several studies based on economic models suggest that vaccination is an economically feasible alternative for the control of bovine neosporosis and at present, there is no commercially available *N. caninum* vaccine, the development of an efficient vaccine would be an important step forward in this research field.

The fact that *N. caninum* is an obligate intracellular parasite suggests that cell-mediated immunity plays a major role in infection control (Innes et al., 2002; Hemphill et al., 2006). A biased Th1 cell-mediated immune response, associated with the production of high levels of cytokines such as IL-12 and IFN-γ, seems to be critical to the host resistance against the infection (Baszler et al., 1999; Long & Baszler, 2000; Ritter et al., 2002). However, this type of response during pregnancy could compromise the viability of the fetus. Contrarily, a Th2 response, which helps maintain pregnancy, could promote the vertical transmission of the parasite (López-Pérez et al., 2008; López-Pérez et al., 2011). Thus, an efficacious vaccine against a parasite such as *N. caninum* should elicit an acquired Th1-type immune response that can control the protozoan infection without compromising pregnancy.

Furthermore, a vaccine against *N. caninum* should be designed according to the required outcome. Depending on the stage of the parasite biological life cycle in which they are developed to act, vaccines may be used to prevent the clinical signs of the disease (i.e. abortion or vertical transmission) or to control or even eradicate the infection at the population level. For instance, the target of a *N. caninum* vaccine may be avoiding the recrudescence of a chronic infection and its consequences. In that case, the vaccine design should focus either on avoiding the conversion process of bradyzoites into tachyzoites or on limiting the parasitaemia resulting from a reactivation, thus protecting against the consequent transplacental transmission. On the contrary, a vaccine aiming at preventing the novo infections associated with epidemic abortion storms should focus on preventing infection due to ingestion of infective oocysts.

In the last few years, several strategies have been implemented to develop vaccines against *N. caninum*, including live, inactivated or new generation recombinant DNA vaccines (Innes et al., 2011). Although new generation vaccines seem to be a promising alternative for the future, those vaccines containing only a few recombinant antigens may

elicit a less effective immune response than vaccines containing antigens from the whole parasite. In fact, most veterinary vaccines available on the market are conventional vaccines based on live or inactivated whole organisms (Meeusen et al., 2007). In general, killed vaccines are not as effective as live ones, but they tend to be more stable and do not pose the risk of reversion to virulence. On the contrary, in terms of protective immunity against protozoan infections, the immune response that live vaccines can elicit is more similar to what would occur during a natural infection (Innes et al., 2011). However, live vaccines have several disadvantages; such as their expensive manufacture costs, their short shelf life, their storage problems and the possibility of reversion to virulence. The use of naturally attenuated organisms as live vaccines against neosporosis is currently under evaluation, showing promising results in terms of protection both in laboratory models and cattle (Miller et al., 2005; Williams et al., 2007). Several studies evidence that some isolates from asymptomatically infected animals may show an attenuated virulence and, therefore, are reasonable candidates for the development of a live vaccine against the disease (Miller et al., 2002; Pereira García-Melo et al., 2010; Regidor-Cerrillo et al., 2010) due to their theoretical safety, which is an extremely important requirement for these vaccines.

In the development of effective vaccines against neosporosis and, in general, against any protozoan, an essential role is played by the selection of parasite antigens and delivery systems that enable appropriate processing by and presentation to the host immune system in order to induce effective innate and adaptative immune responses (Innes & Vermeulen, 2006). On the one hand, some factors related to antigen composition, such as the dose and the type of antigen preparation (whole parasites, soluble extract, total extract, etc.), may determine antigen presentation, modulating the type of elicited immune response (type 1 or type 2). On the other hand, the complexity of the *N. caninum* life cycle, which could imply the presentation of a great stage-specific antigen diversity, emphasizes the possible need to develop preparations with antigens from different life-cycle stages. This approach may be beneficial to interrupt important parasitic processes, such as bradyzoite into tachyzoite conversion, which is related to parasite persistence in the host, or the lytic cycle of the tachyzoite stage during the acute phase of the disease.

Once the candidate antigenic composition is identified, another major challenge for the development of a vaccine is the selection of an adjuvant that can stimulate a protective Th1 immune response. Several adjuvants have been used against neosporosis, mainly to maximize the efficacy of recombinant and inactivated vaccines. However, only a few researchers have compared adjuvant efficacy (Andrianarivo et al., 2000; Baszler et al., 2000; Lunden et al., 2002) and the variety of results observed after immunization with the vaccines is probably attributable to the different types of antigens used. Since adjuvants are critical to the design of non-living vaccines, studies that shed light on their role in protecting against neosporosis are needed.

The aim of the present Doctoral Thesis was to develop vaccines against bovine neosporosis using inactivated and live parasites obtained through conventional technologies. To this end, various vaccine formulations were designed. They were based, on the one hand, on the combination of whole inactivated parasites with different adjuvants and,

on the other hand, on live parasites that were isolated from asymptomatic animals and that could be not only safe, but also capable of eliciting a protective immune response in the host. The safety and efficacy of these vaccine preparations were then tested on well-established animal models of congenital and cerebral neosporosis.

For this purpose, the following objectives were determined.

Objective 1 (Chapters II and III). Development of inactivated whole vaccines against neosporosis.

Sub-objective 1.1. (Chapter II). Influence of adjuvant and antigen dose on the protection induced by an inactivated whole vaccine against *N. caninum* infection in mice.

This chapter investigates the role of three different adjuvants (water-in-oil emulsion -W/O- and aluminum hydroxide plus CpG-ODN -Al/CpG- or plus ginseng extract -Al/G-) and three doses of antigen in the protection against neosporosis. A trial-and-error approach was used to test the efficacy and safety of all the formulations. The efficacy, safety and immunogenicity of the vaccines were evaluated during both the acute and chronic infection phases by means of a BALB/c mouse model of cerebral neosporosis.

Sub-objective 1.2. (Chapter III). Comparative efficacy of immunization with inactivated whole tachyzoites versus a tachyzoite-bradyzoite mixture against congenital and cerebral neosporosis.

This chapter explores the influence of antigens from the tachyzoite and bradyzoite life-cycle stages as well as that of different adjuvants on the prevention of *N. caninum* infection. To this end, different formulations containing previously-tested adjuvants together with inactivated whole tachyzoites or a mixture of whole inactivated tachyzoites and bradyzoites were evaluated. Immunization was elicited by the Nc-Spain7 isolate of *N. caninum,* which shows a high *in vitro* tachyzoite-to-bradyzoite conversion rate. Mouse models of congenital and cerebral neosporosis were used to test out the efficacy and safety of the formulations. Their safety was determined by daily observation and palpation of the mice to detect adverse reactions. The efficacy against congenital infection was calculated by studying neonatal mortality and vertical transmission rates. The efficacy of the vaccines against cerebral infection was mainly measured through the parasite presence in adult mouse brains. In addition, immune responses were determined by means of serological and cytokine expression analysis before and after challenge infection.

Objective 2 (Chapters IV, V and VI). Development of live vaccines against neosporosis by using a naturally attenuated isolate of *N. caninum*.

Sub-objective 2.1. (Chapter IV). Isolation and characterization of a bovine isolate of *N. caninum* with low virulence.

Chapter IV describes the isolation and characterization of a new isolate of *N. caninum,* Nc-Spain 1H, obtained from the brain of a clinically healthy but congenitally infected calf. With the aim of evaluating its virulence and, therefore, its suitability as a safe candidate for live vaccines, the biological behavior of Nc-Spain 1H was studied both *in vitro* and *in vivo*. The tachyzoite yield and viability and tachyzoite-to-bradyzoite conversion rates of this isolate were assessed through *in vitro* experiments. Pathogenicity studies were performed with BALB/c mouse models as follows: a pregnant BALB/c mouse model was used to evaluate the transmission of Nc-Spain 1H to progeny, while a non-pregnant BALB/c mouse was used to measure the capacity of the isolate to cause cerebral infection.

Sub-objective 2.2. (Chapter V). Experimental infection with a low virulence isolate of *N. caninum* at 70 days gestation in cattle did not result in fetopathy.

This chapter evaluates the safety of Nc-Spain 1H after its intravenous inoculation in 70-day-pregnant heifers. Such safety was determined through fetal death in the first trimester of gestation. Complemetarily, immunogenicity of the isolate was measured by examining the elicited humoral and cellular immune responses.

Sub-objective 2.3. (Chapter VI). Evaluation of the protection conferred by a naturally attenuated isolate of *N. caninum* against congenital and cerebral neosporosis.

This section looks into the protective immune response induced against congenital and cerebral neosporosis by immunization with live Nc-Spain 1H tachyzoites in BALB/c mouse models. The safety of the isolate was determined by analyzing the parasite presence in brains after immunization. To assess protective efficacy against congenital neosporosis, neonatal mortality and vertical transmission rates were calculated. The vaccine's efficacy against cerebral neosporosis was studied by checking the presence of *N. caninum* in the brains of adult mice. The cellular and humoral immune responses induced after immunization were examined as well. Additionally, the influence of the immunizing dose on protection against congenital and cerebral infection was evaluated.

Capítulo II

Veterinary Parasitology 175 (2011) 220–229

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Influence of adjuvant and antigen dose on protection induced by an inactivated whole vaccine against *Neospora caninum* infection in mice

Silvia Rojo-Montejo[a], Esther Collantes-Fernández[a], Javier Regidor-Cerrillo[a], Antonio Rodríguez-Bertos[a], Antoni Prenafeta[b], Mercedes Gomez-Bautista[a], Luis M. Ortega-Mora[a,*]

[a] *SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Avda Puerta de Hierro, Ciudad Universitaria s/n, 28040 Madrid, Spain*
[b] *HIPRA, Avda de La Selva s/n, 17170 Amer, Girona, Spain*

ARTICLE INFO

Article history:
Received 5 July 2010
Received in revised form 5 October 2010
Accepted 12 October 2010

Keywords:
Neospora caninum
Inactivated vaccine
Mice
Adjuvant

ABSTRACT

In this study, the protection afforded by a *Neospora caninum* inactivated vaccine formulated with three different adjuvants (water-in-oil emulsion, aluminum hydroxide with CpG oligodeoxynucleotides and aluminum hydroxide with ginseng extract) and three different parasite doses (10^5, 5×10^5 or 10^6 inactivated whole tachyzoites) was evaluated using a mouse model. Mice were immunized subcutaneously twice at three-week intervals with inactivated Nc-Spain 1H tachyzoites and challenged by intraperitoneal inoculation with 10^6 live Nc-1 tachyzoites. The efficacy of the immunization was evaluated in non-pregnant BALB/c mice on days 1 and 5 (acute infection phase) and days 14 and 30 (chronic infection phase) post-challenge. The results showed the ability of water-in-oil emulsion combined with inactivated 5×10^5 tachyzoites to induce protection against neosporosis during the chronic stage, limiting parasite multiplication in the brain. Aluminum hydroxide-ginseng extract and inactivated tachyzoites reduced the number of parasites circulating in the blood during acute phase but failed to limit the establishment of chronic infection. On the other hand, a dose-effect was observed in groups vaccinated with aluminum hydroxide-ginseng extract in which the lesion severity increased as the inactivated tachyzoite dose. This study demonstrates that efficacy can significantly vary depending on the adjuvant, the dose of antigen and the phase of *N. caninum* infection in which the vaccine is tested.

1. Introduction

Neospora caninum is cyst-forming coccidian parasite that is recognized worldwide as a cause of reproductive failure in cattle (Dubey et al., 2007). Neosporosis is generally latent and asymptomatic in non-pregnant cattle, although the consequences of infection in a pregnant cow can be abortion, birth of a weak calf or birth of a clinically healthy but persistently infected calf (Innes et al., 2002). The development of an effective and safe vaccine against bovine neosporosis is of great importance today due to the significance of the economic losses in the dairy and beef industries (Dubey et al., 2007).

Immunization with live tachyzoites has been reported to confer excellent protection in mice (Miller et al., 2005) and cattle (Williams et al., 2007). However, live parasite vaccines can have problems with safety and short shelf-life; thus the development of an inactivated vaccine could be a practical option for the control of bovine neosporosis. In the last few years, antigen extracts of *N. caninum* tachyzoites and recombinant antigens have been tested in mice, displaying some success at inducing a protective immune response (Innes and Vermeulen, 2006).

* Corresponding author. Tel.: +34 913944069; fax: +34 913944098.
E-mail address: luis.ortega@vet.ucm.es (L.M. Ortega-Mora).

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doi:10.1016/j.vetpar.2010.10.028

The use of potent adjuvants that can boost antigen immunogenicity and induce an appropriate immune response is a critical factor in designing inactivated formulations. In particular, it is one of the main goals in devising vaccines against intracellular protozoan infections such as neosporosis, protection from which cell-mediated immunity (CMI) plays a significant role (Innes et al., 2002). Traditional adjuvants such as emulsions and aluminum hydroxide have been widely tested in vaccine trials against several protozoa such as *Plasmodium falciparum* (Coler et al., 2009), *Toxoplasma gondii* (Petersen et al., 1998; Martin et al., 2004) and *Leishmania major* (Tonui et al., 2004). It has been suggested that the mechanism of action of these adjuvants is primarily the slow release of antigen into draining lymph (Brewer and Alexander, 1997). Both types of adjuvant have also been proposed to enhance antigen uptake, activate innate immune pathways and induce local recruitment of immune cells, thereby generating an immunocompetent environment at the injection site (Tritto et al., 2009). Administration of water-in-oil (W/O) emulsions stimulates antibody producing plasma cells and also enhances cellular immune responses (Aucouturier et al., 2001; Aguilar and Rodriguez, 2007). On the other hand, aluminum hydroxide has been associated with the induction of high levels of antibodies and Th2-type responses when used alone (Lindblad, 2004). However, when co-administered with other adjuvants such as oligodeoxynucleotides containing unmethylated CpG motifs (CpG-ODN) or saponins from ginseng, aluminum hydroxide may synergistically enhance the immune response, inducing both Th1- and Th2-type immune responses (Mutwiri et al., 2004; Sun et al., 2008).

Antigen dose has been shown to influence both the type of immune response and the production of cytokines (Hosken et al., 1995), which may influence the efficacy of a vaccine. In neosporosis, no studies focused on antigen dose influence has been carried out, but this factor seems to be important since high parasite doses has been suggested to exacerbate cerebral infections (Baszler et al., 2000).

Because the adjuvant and dose of antigen may influence the development of a protective immune response, the present study was carried out to evaluate different combinations of adjuvants (water-in-oil emulsion and aluminum hydroxide plus CpG-ODN or ginseng extract) and doses of antigen (low, medium, high) for the development of protective efficacy using an inactivated vaccine against *N. caninum* in a BALB/c mouse model.

2. Materials and methods

2.1. Mice

Eight-week-old female BALB/c mice were obtained from a commercial supplier (Harlan Interfauna Ibérica, Spain). They were free of common viral, parasite and bacterial pathogens according to the results of routine screening procedures performed by the manufacturer. Mice were fed *ad libitum*, in a controlled environment with a 12-h light and 12-h dark cycle. All mouse handling procedures complied with EU legislation.

2.2. Parasites and antigen

Two different *N. caninum* isolates were used: Nc-Spain 1H, originally obtained from the brain of a naturally infected asymptomatic calf (Rojo-Montejo et al., 2009b) was used for immunization, and Nc-1 (Dubey et al., 1988) was used for the challenge dose. Both isolates were maintained under the same conditions in a continuous passage of Marc-145 cells as described previously (Perez-Zaballos et al., 2005). Nc-Spain 1H tachyzoites were washed three times in sterile phosphate-buffered saline (PBS, pH 7.4), separated from host cell debris by passing the mixture through a 25-gauge needle, followed by passage through a PD-10 column (Amersham Biosciences, Sweden). The average number of tachyzoites was determined by counting five aliquots using a Neubauer chamber (standard error of the mean 5%). Then, Nc-Spain 1H purified tachyzoites were inactivated with 0.01 M (final concentration) binary ethylenimine (BEI) for a period of 96 h at 4 °C, followed by neutralization with sodium thiosulfate (Andrianarivo et al., 2000).

For challenge, Nc-1 tachyzoites were harvested, and viability was determined by Trypan blue exclusion followed by counting in a Neubauer chamber. The organisms were adjusted to a concentration of 10^6 tachyzoites in a final volume of 200 μl/mouse and used immediately to infect the mice.

Nc-1 tachyzoites for PCR controls were purified as described above, pelleted by centrifugation (600 × *g*, 10 min) and frozen at −80 °C until use. To obtain soluble *N. caninum* protein antigen, purified Nc-1 tachyzoites were suspended in 1 ml of 10 mM Tris–HCl containing 2 mM phenylmethylsulfonyl fluoride (Sigma, USA) and disrupted by ultrasound treatment (Sonifier 450, Branson Ultrasonic, USA) in an ice-bath. Cell debris and unlysed cells were removed by centrifugation (10,000 × *g*, 20 min, 4 °C). Supernatant protein was quantified using the Micro BCA protein assay (Pierce, USA), and then the supernatant was aliquoted and frozen at −80 °C until use.

2.3. Adjuvants

Inactivated whole Nc-Spain 1H tachyzoites were incorporated into a water-in-oil emulsion (adjuvant A), aluminum hydroxide with CpG oligodeoxynucleotides (adjuvant B) or aluminum hydroxide with ginseng extract (adjuvant C). In the adjuvant A, the emulsion was used at a concentration of 104 mg per dose. In adjuvants B and C, aluminum hydroxide was used at a concentration of 1.53 mg per dose, combined with 0.01 mg of CpG oligodeoxynucleotides or 0.4 mg of ginseng extract, respectively. All the adjuvant preparations were developed by HIPRA (Girona, Spain). The immunizing doses were prepared in a final volume of 200 μl per mouse.

2.4. Experimental design and sampling

BALB/c mice were divided into 17 groups, each consisting of 20 animals. Each of the mice was subcutaneously injected with either 10^5, 5×10^5 or 10^6 inactivated whole Nc-Spain 1H tachyzoites incorporated into adjuvant A, B, C

Table 1
Summary of group characteristics.

Groups	Adjuvant	Killed tachyzoites dose	Challenge dose (Nc-1)
1	A	10^5	10^6
2	A	5×10^5	10^6
3	A	10^6	10^6
4	A	PBS	10^6
5	B	10^5	10^6
6	B	5×10^5	10^6
7	B	10^6	10^6
8	B	PBS	10^6
9	C	10^5	10^6
10	C	5×10^5	10^6
11	C	10^6	10^6
12	C	PBS	10^6
13	PBS	10^5	10^6
14	PBS	5×10^5	10^6
15	PBS	10^6	10^6
16	PBS	PBS	10^6
17	PBS	PBS	PBS

or phosphate-buffered saline (PBS). Other groups received adjuvant A, B, C or PBS alone (Table 1). The mice were immunized subcutaneously twice at three-week intervals and sublethally challenged intraperitoneally (i.p.) three weeks after the last immunization with 10^6 live Nc-1 tachyzoites. The animals were monitored daily for the presence of clinical signs of neosporosis. Evaluation of clinical signs was based on previous *N. caninum* infection studies (Atkinson et al., 1999; Eperon et al., 1999; Collantes-Fernandez et al., 2006). Five randomly selected animals from each group were sacrificed on days 1 and 5 (acute infection stage) and days 14 and 30 (chronic infection stage) post-challenge. Blood samples were collected by cardiac puncture in EDTA tubes, centrifuged and plasma was recovered and cryopreserved at −80 °C for antibody analysis. Pelleted EDTA-blood cells were stored at 4 °C until DNA extraction. Brain and lung samples were aseptically recovered and frozen at −80 °C until they were analyzed by PCR. On days 14 and 30 post-challenge, one brain hemisphere was fixed in 10% neutral buffered formalin solution for processing by routine histological methods.

2.5. DNA extraction and ITS1 nested-PCR

A Real Pure Extraction genomic DNA kit (Durviz, Spain) was employed to extract DNA from tachyzoites and 10–20 mg of host tissues, and the Real Pure DNA Extraction SSS (Durviz, Spain) to extract DNA from blood samples according to the manufacturers' protocols. The quantity of DNA was measured spectrophotometrically, and samples were diluted to a final concentration of 40 ng/μl for DNA detection by nested-PCR and quantification by real time-PCR. For detection of parasite DNA, a nested-PCR on the internal transcribed spacer (ITS1) region of *N. caninum* was carried out with four oligonucleotides as described by Buxton et al. (1998). Secondary amplification products were visualized by 1.8% agarose gel electrophoresis and ethidium bromide staining. To avoid false positive reactions, DNA extraction, PCR sample preparation and electrophoresis were performed in separate rooms employing different sets of instruments, aerosol barrier tips and disposable gloves. Moreover, negative control samples were included in each set of DNA extractions and PCR reactions.

2.6. Evaluation of parasite burden by real-time PCR

N. caninum DNA in positive nested-PCR samples was quantified by real-time PCR as described previously (Collantes-Fernandez et al., 2002) using an ABI 7300 Prism Sequence Detector Machine (Applied Biosystems, USA) and the commercial kit Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, UK). Oligonucleotide primers pairs from the *N. caninum* Nc5 sequence that amplify a 76-bp DNA fragment were used to quantify parasite load, and for the quantification of host DNA, specific fragment of 71 bp was amplified from the 28S rRNA gene (Collantes-Fernandez et al., 2002). Samples were run in duplicate in separate tubes. *N. caninum* organisms were quantified by interpolation of Ct values (cycle threshold: the fractional cycle number reflecting a positive PCR result) on a standard curve from DNA equivalent to 10^{-1} to 10^4 tachyzoites. The amount of DNA per sample was normalized by quantification of the 28S rRNA gene, and a standard curve was generated with five-fold serial dilutions of murine DNA quantified by UV spectrophotometry. The data were analyzed with Sequence Detection System Software v.1.6 (Applied Biosystems, USA). Parasite load was expressed as parasite number/μg host DNA.

2.7. Histopathological analysis

Multiple sections of different regions of the brain were examined by routine histological methods. Tissues fixed in 10% neutral formalin and dehydrated through graded alcohols were paraffin embedded, sectioned, and stained with hematoxylin and eosin. Analysis was based on the observation of lesions characteristic of or consistent with *N. caninum* infection in the brain (Lindsay et al., 1995; Collantes-Fernandez et al., 2004). Lesions in the brain were assessed according to the severity of inflammation and the extension of affected tissue in each section, and lesion scores were assigned on a three-point scale according to the following scheme: no lesion (=0); mild meningitis, perivascular cuffing and gliosis (=1); moderate meningitis, perivascular cuffing, gliosis, mild glial nodules and focal granulomas (=2); or severe lesions including meningitis, perivascular cuffing, gliosis, glial nodules and multifocal granulomas (=3) (Pereira Garcia-Melo et al., 2010). The mean of these values was determined for each animal and a median lesion score corresponding to each group was represented.

2.8. Humoral immune responses

N. caninum-specific serum isotypes IgG2a and IgG1 were determined by ELISA. Briefly, 96-well plates were coated with soluble *N. caninum* tachyzoite antigen (0.5 μg in 100 μl/well), and diluted murine serum samples (1:100)

Table 2
Detection of *N. caninum* DNA by nested-PCR in blood, lungs and brain on days 1–5 (a) and days 14–30 post-challenge (b).

Groups	Blood		Lung		Brain	
	a	b	a	b	a	b
1 ($A/10^5$)	9/10[a]	1/10	5/10	0/10	1/10	2/10
2 ($A/5 \times 10^5$)	7/10	0/10	5/10	0/10	1/10	0/10
3 ($A/10^6$)	8/10	0/10	6/10	0/10	1/10	3/10
4 (A/PBS)	8/10	0/10	5/10	1/10	0/10	5/10
5 ($B/10^5$)	8/10	1/10	5/10	2/10	0/10	4/10
6 ($B/5 \times 10^5$)	8/10	0/10	3/10	2/10	0/10	3/10
7 ($B/10^6$)	7/10	0/10	4/10	2/10	1/10	1/10
8 (B/PBS)	8/10	0/10	4/10	0/10	0/10	6/10
9 ($C/10^5$)	5/10	1/10	4/10	3/10	0/10	7/10
10 ($C/5 \times 10^5$)	2/10	1/10	6/10	3/10	1/10	7/10
11 ($C/10^6$)	4/10	1/10	2/10	5/10	0/10	8/10
12 (C/PBS)	8/10	0/10	4/10	0/10	0/10	4/10
13 ($PBS/10^5$)	10/10	1/10	7/10	2/10	2/10	6/10
14 ($PBS/5 \times 10^5$)	9/10	0/10	5/10	4/10	0/10	8/10
15 ($PBS/10^6$)	7/10	0/10	3/10	4/10	1/10	5/10
16 (non-immunized/ challenged control)	8/10	0/10	5/10	1/10	1/10	5/10
17 (non-immunized/non-challenged control)	0/10	0/10	0/10	0/10	0/10	0/10

[a] Fractions represent number of mice positive by nested-PCR/ number of mice tested.

and anti-mouse IgG2a or IgG1 antibody (1:5000; Southern Biotechnology, USA) were used as described previously (Collantes-Fernandez et al., 2006).

2.9. Statistical analysis

No significant differences were found in frequency of parasite detection between the two time points per infection phase. Consequently, to make the statistical analysis more consistent by increasing the number of animals per group, data collected within a phase were pooled (on days 1 and 5 p.i. for acute phase and on days 14 and 30 for chronic phase). All immunized groups were compared to the non-immunized/challenged group to evaluate the protective efficacy of the vaccine. The influence of the adjuvant type and killed *N. caninum* tachyzoites dose was evaluated by comparing the different adjuvants at the same antigen dose and the different antigen doses with the same adjuvant group, respectively.

Frequencies of parasite detection were compared by Fisher's exact test. The parasite load and lesion scores were analyzed using the Kruskal–Wallis test followed by a non-parametric multiple-comparison test. When a statistically significant difference was obtained using the Kruskal–Wallis test but the multiple comparison test failed to reveal it, the results obtained by the Kruskal–Wallis test were preferred as indicated by Morrison (2002). Serological data were compared using one-way ANOVA followed by Duncan's Multiple Range test. All statistical analyses were performed using STATGRAPHICS Plus 4.1 (StatPoint, USA).

3. Results

3.1. Clinical signs

Clinical signs compatible with *N. caninum* infection (inactivity, rough coat and pelvic limb weakness) were observed in the fourth week post-challenge (chronic stage) in very few animals. Specifically, one mouse each from groups 10 ($C/5 \times 10^5$) and 12 (C/PBS) displayed clinical signs.

3.2. Parasite detection in the blood, lungs and brain

The presence of *N. caninum* DNA in blood and lung was more often detected during the acute infection phase (days 1–5 post-challenge), whereas in brain was mainly observed in the chronic infection phase (days 14–30 post-challenge) (Table 2).

Significant differences during acute phase were only observed in parasitaemia. In particular, the immunization with $C/5 \times 10^5$ (group 10) reduced significantly the parasitaemia compared to non-immunized/challenged animals (group 16) ($P < 0.05$). When the adjuvants were compared, the number of animals with parasitaemia was lower in group vaccinated with $C/5 \times 10^5$ (group 10) than in those inoculated with $A/5 \times 10^5$ (group 2), $B/5 \times 10^5$ (group 6) and $PBS/5 \times 10^5$ (group 14) ($P < 0.05$). The effect of antigen dose was observed in groups given adjuvant C. Particularly, parasite presence in blood was lower in animals immunized with $C/5 \times 10^5$ (group 10) than in those inoculated with C/PBS (group 12) ($P < 0.05$).

During chronic stage (days 14–30 post-challenge), parasitaemia was transient in all groups and no significant differences were found. The parasite presence in the lungs was low in most groups. However, when the influence of adjuvant was evaluated, significant increased parasite presence was detected in lungs from the group given $C/10^6$ (group 11) compared with group vaccinated with $A/10^6$ (group 3) ($P < 0.01$). In addition, parasite DNA was more often detected in groups immunized with inactivated tachyzoites plus PBS than in animals vaccinated with adjuvant A either with 5×10^5 or 10^6 inactivated tachyzoites (groups 14, 15 versus 2, 3; $P < 0.05$). In the brain, no pos-

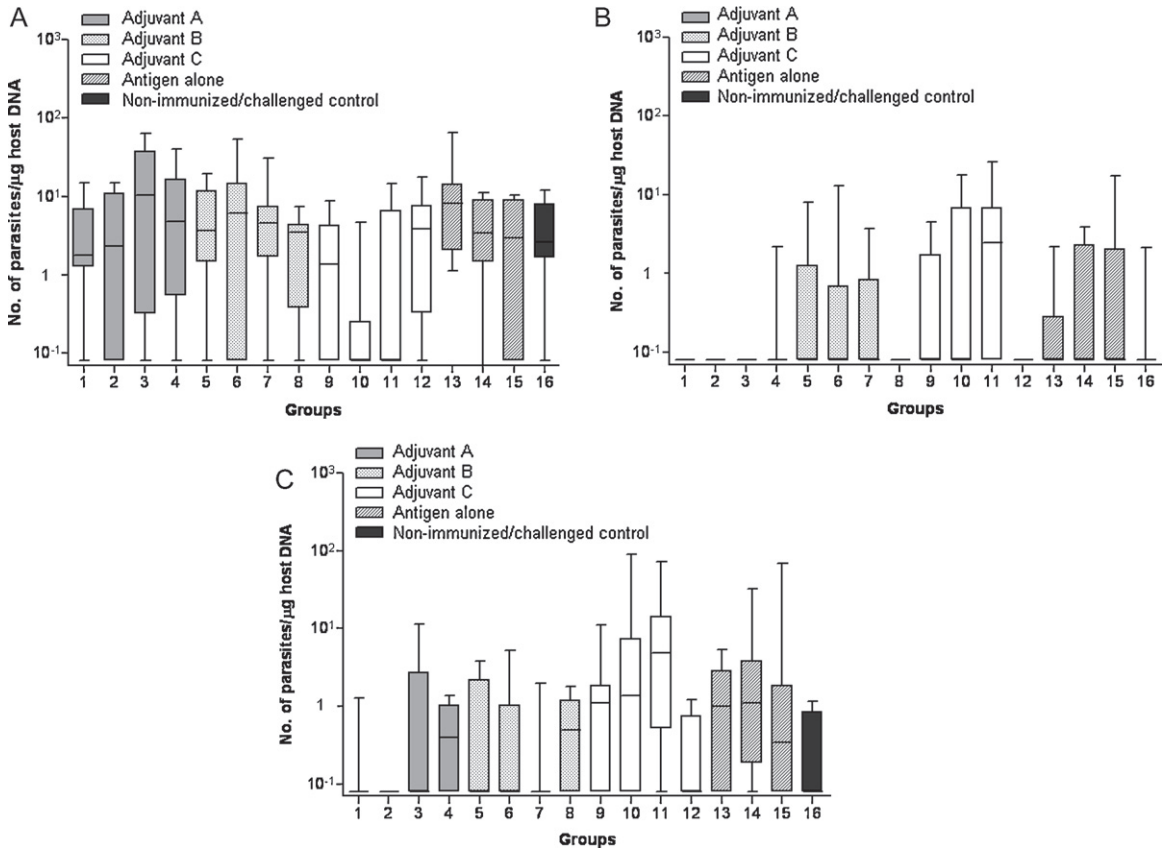

Fig. 1. Box-plot and whiskers graph represents the lower, upper quartiles, median and minimum–maximum of the parasite burden (number of parasites per μg of host DNA) in (A) blood on days 1–5 post-challenge, and (B) lungs and (C) the brain on days 14–30 post-challenge. Mice were s.c. immunized with 10^5 (groups 1, 5, 9 and 13), 5×10^5 (groups 2, 6, 10 and 14) or 10^6 (groups 3, 7, 11 and 15) inactivated whole tachyzoites or no antigen (groups 4, 8 and 12) incorporated into adjuvant A (water-in-oil emulsion), B (aluminum hydroxide-CpG), C (aluminum hydroxide-ginseng extract) or PBS. Taking into account that the *N. caninum* detection limit by real-time PCR is 10^{-1} parasites (Collantes-Fernandez et al., 2002), all positive samples had ≥0.1 parasites. Negative samples (0 parasites) were represented in log scale as <0.1 parasites.

itive animals were found in group 2 ($A/5 \times 10^5$) in which the parasite presence was significantly lower than in non-immunized/challenged animals (group 16) ($P < 0.01$). An adjuvant-dependent effect was detected in groups immunized with adjuvant A or B. Particularly, parasite DNA was demonstrated in a lower number of animals given $A/10^5$ and $A/5 \times 10^5$ than in groups immunized with $C/10^5$, $C/5 \times 10^5$, $PBS/5 \times 10^5$ (groups 1 *vs.* 9; 2 *vs.* 10, 14; $P < 0.05$). Immunization of mice with adjuvant B combined with 10^6 inactivated tachyzoites also significantly reduced the parasite presence compared with group vaccinated with $C/10^6$ (group 11) ($P < 0.05$). The effect of antigen dose was observed in groups given $A/5 \times 10^5$ and $B/10^6$ in which parasite presence was significantly reduced compared with groups vaccinated with the adjuvant-control groups (A/PBS and B/PBS) ($P < 0.05$).

These results indicate that immunization with $C/5 \times 10^5$ lead to the reduction of parasitaemia in the acute infection phase whereas immunization with $A/5 \times 10^5$ efficiently limited the establishment of infection in brain.

3.3. Evaluation of parasite burden

On days 1–5 post-challenge, significant differences were observed in blood and groups vaccinated with adjuvant C which showed a lower parasitaemia level ($P < 0.05$) (Fig. 1A). The immunization with $C/5 \times 10^5$ (group 10) reduced significantly the parasitaemia compared to non-immunized/challenged animals (group 16) ($P < 0.05$). When we determined the adjuvant effect, animals vaccinated with $C/5 \times 10^5$ (group 10) reduced parasitaemia level compared with $B/5 \times 10^5$ (group 6) and $PBS/5 \times 10^5$ (group 14) ($P < 0.01$) (Fig. 1).

On days 14–30 post-challenge, animals immunized with adjuvant C and 10^6 inactivated tachyzoites had greater parasite loads in the lungs (group 11 vs. group 3; $P < 0.05$) (Fig. 1B). In the brain, mice vaccinated with $C/10^6$ had an even higher parasitic load than the non-immunized/challenged animals ($P < 0.05$). The effect of adjuvant on the reduction of brain parasite loads was observed in groups vaccinated with adjuvant A or B ($P < 0.01$) (Fig. 1C). Specifically, groups vaccinated with

A/5×10^5 or B/10^6 showed significantly lower parasite burdens than those immunized with adjuvant C/5×10^5, C/10^6 and PBS/5×10^5 (2 vs. 10, 14; 7 vs. 11; $P < 0.01$). A dose-dependent effect was observed in groups immunized with adjuvant C in which an increase in the parasite load was associated with a higher dose of inactivated tachyzoites (11 vs. 12, $P < 0.01$).

Altogether, these data show the ability of adjuvant C and inactivated tachyzoites to reduce the number of parasites circulating in the blood during acute phase but not to limit the establishment of chronic infection in brain. On the other hand, immunization with adjuvant A or B and inactivated tachyzoites displayed the lowest brain parasite burden in the chronic infection phase.

3.4. Histopathology

In groups vaccinated with inactivated tachyzoites and adjuvant C and PBS, histopathological examination of brain tissue from mice sacrificed on days 14–30 post-challenge revealed the presence of large severe necrotic foci with mononuclear infiltrates. A few animals, specifically from the PBS/5×10^5 and C/10^6 groups, showed foci of dystrophic calcification in the necrotic areas. Animals vaccinated with adjuvants A or B and non-immunized/challenged animals had mild to moderate microgliosis, perivascular cuffs or no pathology. The statistical analysis showed that mice vaccinated with adjuvant C or PBS combined with higher tachyzoite doses had higher lesion severity scores than the non-immunized/challenged animals (group 11 and group 14 vs. group 16; $P < 0.01$). Regarding adjuvant influence, immunization with adjuvants A or B combined with higher inactivated parasite doses reduced the severity of lesions compared with animals immunized with PBS (groups 2, 6 vs. 14) or adjuvant C (groups 3, 7 vs. 11) ($P < 0.01$) (Fig. 2). In addition, a dose-effect was observed in groups vaccinated with adjuvant C in which the lesion severity increased as the inactivated tachyzoite dose increased (group 9, 12 vs. group 11; $P < 0.01$).

Taken together these results show that adjuvant C or PBS plus high doses of inactivated tachyzoites not only do not confer protection but also exacerbate the cerebral neosporosis.

3.5. Humoral immune response

Production of IgG1 was predominant during the experiment in the vaccinated groups.

On days 1–5 post-challenge (Fig. 3A), adjuvant A with 5×10^5 or 10^6 inactivated tachyzoites induced higher levels of IgG1 and IgG2a isotypes than mice vaccinated with adjuvants B, C or PBS plus inactivated tachyzoites and than non-immunized/challenged animals ($P < 0.05$). An increase in antibody concentration was also observed in animals vaccinated with PBS and inactivated tachyzoites (groups 14 and 15) compared with adjuvants B and C ($P < 0.0001$). In addition, a parasite dose effect was observed since the higher inactivated tachyzoites doses stimulated higher IgG1 and IgG2a values ($P < 0.0001$).

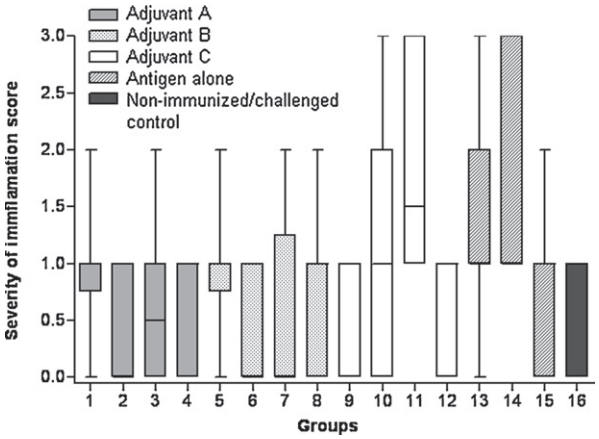

Fig. 2. Box-plot and whiskers graph represents the lower, upper quartiles, median and minimum–maximum of the lesion severity score (nil = 0, mild = 1, moderate = 2, and severe = 3) in the brain on days 14–30 post-challenge. Mice were s.c. immunized with 10^5 (groups 1, 5, 9 and 13), 5×10^5 (groups 2, 6, 10 and 14) or 10^6 (groups 3, 7, 11 and 15) inactivated whole tachyzoites or no antigen (groups 4, 8 and 12) incorporated into adjuvant A (water-in-oil emulsion), B (aluminum hydroxide-CpG), C (aluminum hydroxide-ginseng extract) or PBS.

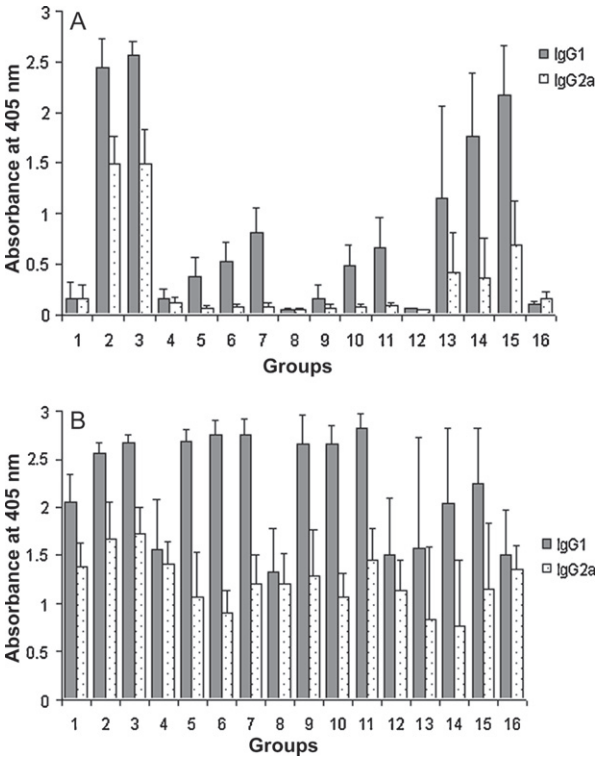

Fig. 3. Levels of anti-*N. caninum*-specific IgG2a and IgG1 in sera of BALB/c mice immunized subcutaneously twice at three-week intervals. Blood samples were collected from the mice 1–5 days (A) and 14–30 days after the challenge (B). The specific IgG2a and IgG1 levels were assessed using ELISA. Bars represent the average absorbance value at 405 nm and standard error bars represent the standard deviation.

On days 14–30 post-challenge (Fig. 3B), mice vaccinated with inactivated tachyzoites with adjuvant A, B, C and PBS developed higher IgG1 levels than non-immunized/challenged animals during chronic infection ($P < 0.05$). Additionally, mice given adjuvants A, B and C displayed higher IgG1 values than groups inoculated with PBS as well as either 5×10^5 or 10^6 inactivated tachyzoites and control adjuvant groups ($P < 0.01$). However, IgG2a levels were only significantly higher in groups vaccinated with A/10^6 compared with A/10^5 or the adjuvant control group (A/PBS) ($P < 0.05$).

In summary, all challenged groups had higher concentration of IgG1 than IgG2a. Furthermore, immunization with adjuvant A induced the highest antibody levels.

4. Discussion

In designing an inactivated vaccine formulation, the choice of appropriate adjuvants and antigen doses is crucial for a successful vaccination program, and is essentially empiric. Therefore, trial-and-error studies are a useful approach to select vaccine candidates with acceptable safety and proven efficacy. Here, we performed a concurrent study of several adjuvants formulated with the same antigen at different doses, evidencing the role of these factors on protection against *N. caninum* infection.

In addition, the use of a laboratory model for testing vaccine formulations is necessary for the selection of effective protocols prior to their use in cattle. We used a BALB/c mouse model previously developed in our laboratory, in which experimental infections with Nc-1 were characterized by an early phase, during which parasitaemia and parasite DNA was detected mainly in lungs, and a chronic stage with parasite presence in the brain (Collantes-Fernandez et al., 2006). Nc-1 isolate has been widely used as the challenge isolate in numerous vaccine studies (Lindsay et al., 1999; Lunden et al., 2002; Cannas et al., 2003; Debache et al., 2008; Ribeiro et al., 2009). Inoculation of high doses of Nc-1 tachyzoites in mice has been observed to be lethal, inducing high morbidity and mortality and severe lesions consistent with acute neosporosis (Alaeddine et al., 2005; Ramamoorthy et al., 2006). In order to establish a latent stage of the infection characterized by low mortality, the presence of the parasite in the brain and encephalitis as the main lesion (Dubey and Lindsay, 1996; Collantes-Fernandez et al., 2004), a sublethal challenge dose was utilised. On the other hand, in an attempt to avoid the use of isolates maintained *in vitro* for several years, we used the new isolate Nc-Spain 1H (Rojo-Montejo et al., 2009a,b) with low passage number, because culturing could select for variant parasite populations that do not include antigens or present them incorrectly (Miller et al., 2005). Inactivated whole Nc-Spain 1H tachyzoites were employed to deliver to the immune system with either *N. caninum* organelles or membrane antigens accessible during intracellular or extracellular phases of the infection. Furthermore, inactivated tachyzoites might be a good candidate to use as antigen in vaccine trials since intact inactivated tachyzoites have been shown to induce a greater increase in the number of IFN-γ producing NK cells compared with sonicated soluble antigens (Klevar et al., 2007). Nc-Spain 1H tachyzoites were inactivated with BEI, a chemical method shown to cause fewer epitope changes to the antigen than other treatments (Blackburn and Besselaar, 1991) and that have previously been used in a *N. caninum* inactivated vaccine formulation (Andrianarivo et al., 2000).

Concerning adjuvants, various strategies were chosen. The first approach was to test a standard water-in-oil emulsion (adjuvant A) widely used in ruminants, which primarily acts as a depot. On the other hand, we employed the co-administration of aluminum hydroxide with novelty adjuvants such as CpG-ODN (adjuvant B) or ginseng extract (adjuvant C), which are able to modify the cytokine network. CpG-ODN is a potent activator of the innate immune response that subsequently amplifies the antigen-specific immune response. The saponins from ginseng extract have been reported to have adjuvant properties and appear to exert a number of effects on the immune system, including enhancing lymphocyte proliferation and stimulating the secretion of a broad range of cytokines (Rivera et al., 2005; Sun et al., 2007; Song and Hu, 2009). The combination of aluminum hydroxide with immunostimulatory substances may synergistically enhance the immune response boosting both cellular (Th1) as well as humoral (Th2) immune responses (Davis et al., 1998; Rivera et al., 2003; Sun et al., 2008).

The results of this study demonstrated that the protective efficacy varied depending on the adjuvant and the stage of *N. caninum* infection in which the vaccine was tested. Thus, water-in-oil emulsion (adjuvant A) combined with whole inactivated tachyzoites induced protection against neosporosis during the chronic stage. Specifically, in mice vaccinated with adjuvant A and 5×10^5 inactivated tachyzoites, no detectable parasite DNA and a reduction in the severity of lesions in the brain were found. It is unclear what immunological mechanisms have been developed by this adjuvant in the protection against cerebral neosporosis. The depot effect proposed as a mechanism of action of W/O emulsions probably induced the significant increase of antibody production observed in mice immunized with adjuvant A. W/O-mediated protection was associated with predominant IgG1 but also high IgG2a levels, suggesting that a Th1/Th2 balance could confer protection against cerebral disease. Debache et al. (2008) also observed reduced cerebral infection in mice after immunization with other W/O emulsions such as Freund's incomplete adjuvant mixed with recNcROP protein but in contrast to the present results, the protection was associated with predominant IgG2a levels.

Regarding adjuvant B, the properties of CpG plus aluminum hydroxide combination have been previously demonstrated in vaccine trials against other protozoa (Near et al., 2002; Su et al., 2003). However, no studies have yet been conducted to evaluate the potential of this combination in neosporosis and only the immunostimulatory effect of CpG-ODN has been recently tested against *Neospora*-infection (Ribeiro et al., 2009). In this previous study, the protection induced by CpG-adjuvanted preparations against cerebral infection was primarily influenced by the antigen choice (lysate antigen and excreted-secreted antigens). In the present study, immunization with adjuvant

B and 10^6 inactivated tachyzoites reduced parasite presence and burden in brain but significant protection was not observed against non-immunized/challenged group. The partial control of parasite infection induced by adjuvant B constitutes a promising result and is motivation for future studies.

On the other hand, immunization of BALB/c mice with whole inactivated tachyzoites and aluminum hydroxide-ginseng extract (adjuvant C) significantly reduced acute parasitaemia but failed to protect during the chronic stage. Since the degree of parasitaemia is likely to be an important factor in the outcome of *N. caninum* infection in pregnant cattle (Innes et al., 2002; Staska et al., 2003), vaccination with the adjuvant C could control the vertical transmission of the parasite during pregnancy, as it would reduce the parasitaemia and a lower level of parasites would invade the placenta and foetus. However, mice immunized with adjuvant C showed higher brain parasite burden and lesion severity than non-immunized/challenged animals. In contrast, mice immunized with adjuvant C alone had similar parasite levels to non-immunized/challenged animals, showing that the adverse results seen in mice vaccinated with adjuvant C combined with inactivated tachyzoites could be mainly due to the parasite antigen inoculated. Conversely, our results disagree with a previous study in which the level of parasitaemia appeared to correlate well with disease severity and cerebral parasite loads (Pinitkiatisakul et al., 2008). Immunization with NcSRS2-iscoms decreased the level of parasitaemia, which probably reduced the number of parasites reaching the brain, and this could be the reason for the reduction in brain parasite load and clinical symptoms. The reason why adjuvant C combined with parasite antigen reduced the levels of parasitaemia but which was unsuccessful in protecting during the chronic stage of *N. caninum* infection is unknown. Probably, the immune response induced against rapidly replicating tachyzoites during acute infection could have precipitated parasite survival strategies to evade the immune response. Ginseng extracts have a broad range of immunological activities, including the improvement of phagocytic activity of immune cells (Song and Hu, 2009). Co-administration of ginseng extract and aluminum hydroxide with inactivated tachyzoites may have facilitated the internalization of parasites by migratory cells of the immune system without their neutralization, favouring their rapid dissemination to multiple organs including immunoprivileged tissues such as the brain. Leukocyte trafficking to disseminate intracellular parasites via a Trojan horse-type mechanism has been postulated for other apicomplexan parasites such as *T. gondii* (Lambert et al., 2006).

Unexpectedly, immunization with parasite antigen plus adjuvant B or C induced a low antibody response with a dominance of the IgG1 subclass. The precise immune modulation induced by the adjuvants employed here could not be determined by means of the analysis of *N. caninum*-specific IgG1 and IgG2a production due to a lack of correlation between the protection level and the humoral immune response detected. Therefore, more research is needed into the mechanisms by which protective immunity can be induced by these formulations.

Understanding how antigen dose influences vaccine efficacy is also important for designing vaccines. To date, several immunization schedules have been tested in *N. caninum* inactivated vaccine trials (Baszler et al., 2000; Lunden et al., 2002; Cannas et al., 2003; Ribeiro et al., 2009) although dose-related effects in inactivated formulations have not been assessed. In the present study, three antigen doses (10^5, 5×10^5 and 10^6 inactivated tachyzoites) were tested. The serological response demonstrated a dose-related immunogenicity in all of the immunized groups. However, with respect to protection, we only observed dose-dependent effects in animals immunized with adjuvant C in the chronic stage, in which parasite loads and lesion severity increased in the target organs as did the antigen dose. Similar findings were found after the use of 50 μg of soluble *N. caninum* tachyzoite antigen that resulted in an exacerbation of the infection in mice (Baszler et al., 2000), whereas immunization of mice with low doses of crude lysate such as the equivalent of 10^5 tachyzoites (approximately 5 μg) or 15 μg reduced congenital transmission and protected completely against cerebral infection, respectively (Liddell et al., 1999; Cannas et al., 2003). Moreover, immunization with inactivated tachyzoites and PBS also enhanced susceptibility in the chronic phase, even though we did not find a significant dose-related effect. Previously, it was reported that mice immunized with parasite lysate alone or excreted-secreted antigens were unprotected and appeared even more susceptible than the control mice, independently of the inoculated parasite dose (Lindsay et al., 1999; Lunden et al., 2002; Ribeiro et al., 2009).

In summary, data from this study demonstrated that the choice of adjuvant is crucial to the efficacy of the inactivated vaccine, but the efficacy can also vary depending on antigen dose and the stage of *N. caninum* infection in which the vaccine is tested. Aluminum hydroxide-ginseng extract with 5×10^5 inactivated tachyzoites significantly reduced acute parasitaemia, whereas the highest protection efficacy during the chronic stage was observed with the water-in-oil emulsion with 5×10^5 inactivated tachyzoites, which limited parasite multiplication in brain. This study primarily focused on the efficacy of different vaccine formulations in a laboratory model. We consider this trial-and-error approach a valuable tool for the preliminary selection of promising vaccine protocols against *N. caninum* infection. Further studies are necessary to test the efficacy against congenital neosporosis and for comprehensively evaluating the precise immune modulation induced by these formulations.

Acknowledgements

We would like to thank Vanesa Navarro for her excellent technical assistance and Ricardo García Mata for statistical advice. We are grateful to the SALUVET group for their collaboration in this study. This work was supported by a PETRI 95-0777.OP project from the Ministry of Education and Science of Spain and HIPRA. The Nc-Spain 1H isolate of *N. caninum* has been patented by the SALUVET group and HIPRA.

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Capítulo III

Comparative efficacy of immunization with inactivated whole tachyzoites versus a tachyzoite-bradyzoite mixture against neosporosis in mice

S. ROJO-MONTEJO[1], E. COLLANTES-FERNÁNDEZ[1], S. RODRÍGUEZ-MARCOS[1], F. PÉREZ-ZABALLOS[1], I. LÓPEZ-PÉREZ[1], A. PRENAFETA[2] *and* L. M. ORTEGA-MORA[1]*

[1] *SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain*
[2] *HIPRA, Avda de La Selva 135, Amer 17170, Girona, Spain*

(*Received 12 April 2011; revised 3 June 2011; accepted 15 June 2011*)

SUMMARY

The worldwide economic impact of *Neospora caninum* infection has caused the development of effective vaccines to become one of the main goals in the field of neosporosis research. In this study, the protection conferred by antigens from inactivated whole tachyzoites (TZ) and a tachyzoite-bradyzoite mixture (TZ-BZ) of *N. caninum* (Nc-Spain7 isolate) incorporated into a water-in-oil emulsion (W/O) and aluminium hydroxide-ginseng extract (Al/G) was evaluated in mouse models of congenital and cerebral *N. caninum* infection. Immunization with TZ-BZ induced congenital and cerebral neosporosis exacerbation that was mainly characterized by reduced neonatal median survival time and increased parasite presence in adult mouse brains. The immune response of mice immunized with TZ-BZ was characterized by an increase in IFN-γ expression prior to challenge and an increase in IL-4 expression accompanied with significantly higher levels of antibodies against 2 recombinant bradyzoite-specific proteins (rNcSAG4 and rNcBSR4) after challenge. Immunization with TZ in W/O significantly reduced neonatal mortality, vertical transmission as well as parasite presence in adult mouse brains and induced a strong humoral immune response. The current study demonstrates the critical role of stage-specific antigens and adjuvants on the development of effective inactivated vaccines for the prevention of *N. caninum* infection.

Key words: *Neospora caninum*, inactivated vaccine, tachyzoite, bradyzoite, mice.

INTRODUCTION

The tissue-cyst forming protozoan parasite *Neospora caninum* has emerged as an important cause of reproductive failure in cattle worldwide, leading to significant economic losses in beef and dairy cattle industries (Dubey *et al*. 2007). In cattle, *N. caninum* is able to persist in the brain of an immunocompetent host in a cyst form containing slowly dividing bradyzoites (BZ). The cysts remain quiescent while awaiting an appropriate immunological scenario wherein they switch into fast replicating tachyzoites (TZ). In pregnant animals, TZ can disseminate and cause potentially fatal disease, resulting in abortion, birth of a weak calf or birth of a clinically healthy but persistently infected calf (Buxton *et al*. 2002; Innes *et al*. 2002).

Epidemiological evidence confirming the protective immunity against vertical transmission and abortion in some *N. caninum*-infected cows makes immunoprophylaxis a feasible alternative for control of the disease (Innes *et al*. 2002; Reichel and Ellis, 2006; Dubey *et al*. 2007). In recent years, most vaccine trials against *N. caninum* have focused on molecules from TZ in the form of live, inactivated or recombinant antigens, principally to control the acute phase of infection (Liddell *et al*. 1999; Lunden *et al*. 2002; Reichel and Ellis, 2009; Ribeiro *et al*. 2009). Using vaccines containing parasite antigens from different life-cycle stages could be a promising strategy to improve the protection conferred by vaccines against neosporosis. Recently, we evaluated the role of the recombinant bradyzoite-specific SAG4 (rNcSAG4) protein of *N. caninum* in the protection against chronic and congenital infection in mice (Aguado-Martínez *et al*. 2009*a*). This study revealed that immunization with rNcSAG4 failed to protect against parasite infection. However, a slight yet significant delay in the death of pups in the rNcSAG4-vaccinated group was observed, motivating future studies to evaluate the effect of vaccines made from bradyzoite antigens. To this end, a mixture of whole *Neospora* TZ and BZ containing native organelle and membrane antigens from both stages could be a good candidate for vaccine development not only against the rapid dissemination of parasite during the acute infection phase but also

* Corresponding author: SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040-Madrid, Spain. Tel: +34 913944069. Fax: +34 913944098. E-mail: luis.ortega@vet.ucm.es

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doi:10.1017/S0031182011001156

against the establishment of persistent *N. caninum* infection.

We previously observed that immunization with whole inactivated TZ conferred protection against acute and chronic neosporosis in non-pregnant BALB/c mice when combined with appropriate adjuvants and antigen doses (Rojo-Montejo *et al*. 2011). Specifically, a water-in-oil emulsion (W/O) adjuvant containing 5×10^5 inactivated whole TZ reduced parasite presence in the brain during the chronic stage of infection, whereas aluminium hydroxide co-administrated with ginseng extract (Al/G) containing 5×10^5 inactivated whole TZ significantly reduced acute parasitaemia. These results suggest that the W/O may control the establishment of infection in the brain, whereas the Al/G may control the vertical transmission of the parasite to progeny by reducing maternal parasitaemia and therefore the number of parasites reaching the placenta and fetus.

The objective of the present study was to investigate the protective efficacy of previously tested vaccine preparations containing inactivated whole *N. caninum* TZ as well as a new formulation of a tachyzoite-bradyzoite mixture (TZ-BZ) combined with W/O or Al/G against congenital and cerebral neosporosis with both pregnant and non-pregnant BALB/c mouse models (López-Pérez *et al*. 2006, 2008).

MATERIALS AND METHODS

Parasite culture

Culture of N. caninum *zoites for immunization*. The *N. caninum* Nc-Spain7 isolate, recently obtained from the brain of an asymptomatic but congenitally infected calf (Regidor-Cerrillo *et al*. 2008), was used for immunization. TZ were grown by continuous passage in MARC-145 cells following standard procedures (Pérez-Zaballos *et al*. 2005). TZ for vaccine formulations were harvested 3·5 days post-infection. For formulations containing TZ-BZ, *in vitro* stage conversion was induced by treatment of Nc-Spain7 tachyzoite-infected cultures with 70 μM sodium nitroprusside (SNP) for up to 7 days (Risco-Castillo *et al*. 2004). Zoites were harvested from tissue culture on day 7 post-stress. Both TZ and TZ-BZ cultures were purified by washing 3 times in sterile phosphate-buffered saline (PBS, pH 7·4) and separated from host cell debris by passing the mixture through a 25-gauge needle following passage through disposable PD-10 desalting columns (GE Healthcare, Buckinghamshire, UK) (Hemphill *et al*. 1996). Cell-free Nc-Spain7 zoites were counted by Trypan blue exclusion followed by counting in a Neubauer chamber, adjusted to a final concentration of 5×10^7 zoites/ml and immediately inactivated as described below in the *Vaccine formulations* section.

Conversion measurement assay. Seven days after stress treatment, the tachyzoite-to-bradyzoite conversion rate was measured by double immunofluorescence (Risco-Castillo *et al*. 2004). Identification of TZ and BZ was performed by labelling parasites with antibodies directed against the immunodominant *N. caninum* tachyzoite surface antigen NcSAG1 (Fuchs *et al*. 1998) and with antiserum against *T. gondii* bradyzoite antigen 1 (BAG1), which exhibits cross-reactivity with *N. caninum* BZ (McAllister *et al*. 1996). Cover-slips were labelled with a monoclonal mouse antibody directed against the tachyzoite surface antigen NcSAG1 (αSAG1) (1:2000) and a polyclonal rabbit antiserum against the intracytoplasmic bradyzoite antigen BAG1 (αBAG1) (1:100) (Risco-Castillo *et al*. 2004). Antibody binding was observed with an inverted fluorescence microscope (Model TE200 Nikon, 100× oil-immersion objective). The conversion rate of TZ to BZ was calculated by random counting of 10 fields per cover-slip and comparing the percentage of BAG1-positive zoites versus total zoites. Double immunofluorescence revealed a TZ-to-BZ conversion rate around 30%, which was used for vaccine formulations containing TZ-BZ antigen.

Parasite culture for challenge. Tachyzoites of the Nc-Liverpool isolate (Barber *et al*. 1995) were maintained *in vitro* by continuous passage in MARC-145 cells using standard procedures (Pérez-Zaballos *et al*. 2005). For *N. caninum* challenge, TZ were prepared following described procedures (López-Pérez *et al*. 2006, 2008) at the required dose of 2×10^6 TZ in a final volume of 200 μl per mouse and used immediately to infect mice.

Vaccine formulations

Purified Nc-Spain7 TZ and TZ-BZ mixtures were inactivated with 0·01 M (final concentration) of binary ethylenimine for a period of 96 h at 4 °C, followed by neutralization with sodium thiosulphate (Andrianarivo *et al*. 2000). Inactivated whole Nc-Spain7 zoites were incorporated into the W/O or Al/G. The W/O was used at a concentration of 104 mg per dose. In the adjuvant Al/G, aluminium hydroxide was used at a concentration of 1·53 mg per dose combined with 0·2 mg of ginseng extract. Both adjuvant preparations were developed by HIPRA (Girona, Spain). The immunizing doses were prepared in a final volume of 200 μl per mouse.

Immunization assay and sample collection

All mouse handling procedures complied with EU legislation. Eight-week-old female BALB/c mice (Harlan Interfauna Ibérica, Barcelona, Spain) were randomly divided into 10 groups. Each mouse was

Table 1. Summary of group characteristics

Group	Adjuvant	Parasite antigen	Challenge dose (Nc-Liv)
1[a]	W/O	TZ	2×10^6
2[b]	W/O	TZ-BZ	2×10^6
3[a]	W/O	PBS	2×10^6
4[a]	Al/G	TZ	2×10^6
5[b]	Al/G	TZ-BZ	2×10^6
6[a]	Al/G	PBS	2×10^6
7[a]	PBS	TZ	2×10^6
8[b]	PBS	TZ-BZ	2×10^6
9a, 9b[c]	—	—	PBS
10a, 10b[c]	—	—	2×10^6

[a] Mice were immunized with *N. caninum* whole TZ incorporated into W/O, Al/G or PBS in groups of 22 mice.
[b] Mice were immunized with the *N. caninum* whole TZ-BZ mixture incorporated into W/O, Al/G or PBS. The number of mice per group was expanded to 32 mice to make the statistical analysis more consistent by increasing the number of pregnant mice per group.
[c] Non-immunized/non-challenged (group 9) and non-immunized/challenged (group 10) mice were included at both time-points (subgroups a and b) of the experiment to ensure the reproducibility of the experimental design. The number of mice in subgroups a and b was 22 and 32, respectively.

injected subcutaneously twice at 3-week intervals with 5×10^5 inactivated whole *N. caninum* zoites (TZ or TZ-BZ) incorporated into the W/O, the Al/G or PBS. Other groups received the W/O, the Al/G or PBS alone (Table 1). For logistical reasons, the experiment was carried out at 2 different times. First, vaccine formulations containing *N. caninum* TZ were inoculated. Next, mice were immunized with *N. caninum* TZ-BZ. Non-immunized/non-challenged (group 9) and non-immunized/challenged (group 10) mice were included at both time-points (subgroups a and b) of the experiment to ensure the reproducibility of the technique. Three weeks after the booster immunization, BALB/c mice were mated for 96 h following synchronization of oestrus using the Whitten effect (Whitten, 1957). Day 0 of pregnancy was defined as the first day that females were housed with males. The challenge infection was achieved by subcutaneously inoculating animals with 2×10^6 Nc-Liverpool TZ at mid-gestation (between days 6 and 10 of gestation). Pregnant animals were housed individually and allowed to carry their pregnancy to term. Pups were evaluated daily from birth to day 30 post-partum (PP) for congenital neosporosis (López-Pérez *et al*. 2008). Dams and non-pregnant mice were evaluated for chronic infection until day 30 PP and day 30 post-challenge, respectively (Collantes-Fernández *et al*. 2006; López-Pérez *et al*. 2008). Brains and lungs from neonates and brains from adult mice were removed aseptically and frozen at −80 °C until required for DNA extraction. Samples from some progeny could not be collected due to cannibalism by the dams. Immune responses were evaluated in 5 randomly selected mice from each group prior to the challenge (2 days after booster) and in dams at the chronic phase (day 30 PP). The number of pregnant mice analysed at this time-point (day 30 PP) varied between 5 and 10 animals per group; the number of pregnant mice analysed was related to the number of dams that survived until the end of the experiment. To measure the humoral immune response, blood samples were collected by cardiac puncture, and the recovered sera were aliquoted and cryopreserved at −80 °C until serological analysis. For measurements of cytokine expression, spleen samples from each group were pooled (0·05 g of spleen tissue per mouse) and cryopreserved at −80 °C until processed as described below.

Parameters evaluated for safety and efficacy

The safety of different formulations was determined by daily observation of mice for adverse reactions and by palpation for the presence of nodules at the inoculation site on day 10 after the booster vaccination. Vaccine efficacy studies were performed using both pregnant and non-pregnant BALB/c mouse models as previously described (Aguado-Martínez *et al*. 2009*a*; Rojo-Montejo *et al*. 2011). In the pregnant mouse model, data on litter size, hebdomadal mortality, temporal evolution and the rate of neonatal mortality and vertical transmission were collected. Litter size was defined as the number of pups delivered per dam. Hebdomadal mortality was defined as the number of full-term dead pups at the time of birth and those that died between birth and day 2 PP. Neonates were examined daily for morbidity and mortality. Neonatal mortality was defined as the number of dead pups from day 2 to day 30 PP. Temporal evolution of neonatal mortality was evaluated using Kaplan-Meier survival curves. Finally, vertical transmission of *N. caninum* was identified by the presence of parasites in the lungs or brains of pups that died within 48 h (hebdomadal mortality) and pups born alive. Vaccine efficacy against the chronic *N. caninum* infection phase was studied in dams and non-pregnant mice by checking clinical signs compatible with neosporosis, mortality and the presence of *N. caninum* DNA in the brain.

DNA extraction and nested PCR

Tachyzoites for PCR controls were prepared as previously described (López-Pérez *et al*. 2006). The Real Pure Extraction genomic DNA kit (Durviz, Valencia, Spain) was used to extract DNA from 10–20 mg of each host tissue and 10^7 *N. caninum* tachyzoites according to the manufacturer's instructions. Amounts of DNA were measured spectrophotometrically, and samples used for DNA

detection by nested PCR were diluted to a final concentration of 50 ng/μl. For the detection of parasite DNA, nested PCR on the internal transcribed spacer (ITS1) region of *N. caninum* was performed with 4 oligonucleotides as described by Buxton *et al*. 1998). Secondary amplification products were visualized by 1·8% agarose gel electrophoresis and ethidium bromide staining. To avoid false positives, DNA extraction, PCR sample preparation and electrophoresis were performed in separate rooms employing different sets of instruments, aerosol barrier tips and disposable gloves. Moreover, negative control samples were included in each set of DNA extractions and PCR reactions.

Analysis of the humoral immune response

Serum levels of *N. caninum*-specific IgG1 and IgG2a were measured as identifiers of Th2 and Th1 immune responses, respectively, with an ELISA based on *N. caninum* soluble tachyzoite antigens (Long *et al*. 1998; Collantes-Fernández *et al*. 2006). Specific IgG responses developed against different life-cycle stages were measured by ELISA based on the stage-specific recombinant NcGRA7 and NcSAG4 proteins according to previously described methods (Aguado-Martínez *et al*. 2009*a*,*b*). Similarly, a recombinant NcBSR4 protein-based ELISA was developed to measure serum levels of specific IgG against NcBSR4 protein. The dense granule protein NcGRA7 is an immunogenic antigen that is highly associated with active replication of the parasite (Jenkins *et al*. 1997; Aguado-Martínez *et al*. 2008). Therefore, we used recombinant NcGRA7 (Álvarez-García *et al*. 2007) as an antigen to evaluate the antibody response developed against TZ. The recently described *N. caninum* NcSAG4 (Fernández-García *et al*. 2006) and NcBSR4 (Risco-Castillo *et al*. 2007) proteins are stage-specific antigens expressed at early and late bradyzoite stages, respectively. We employed both of them to measure the humoral immune response specifically developed against this slowly dividing parasite stage. The ELISA results were expressed as a relative index percentage (RIPC) using the following formula: RIPC = (OD_{405} sample-OD_{405} negative control)/(OD_{405} positive control-OD_{405} negative control) × 100, where OD is the mean value of the optical density. For recombinant protein-based ELISA (GRA7, SAG4 and BSR4), the threshold value arbitrarily discriminating between 'positive' and 'negative' (cut-off) was defined by adding 3 standard deviations to the mean A_{405} value of sera from non-immunized/non-infected mice.

Analysis of cytokine expression

Cytokine expression was evaluated in spleen by real-time RT-PCR as previously described (López-Pérez *et al*. 2010). Briefly, pooled spleens (0·05 g per mouse) were homogenized in the commercial TRI REAGENT (Sigma, St Louis, MO, USA) by a Polytron PT1600E homogenizer (Kinematica, AG, Lucerne, Switzerland). Total RNA was extracted following the manufacturer's instructions, and the integrity was checked by agarose gel electrophoresis. cDNA synthesis was performed with SuperScript II Reverse Transcriptase (Invitrogen, Paisley, UK) following the manufacturer's recommendations. The primer sequences used for the amplification of interferon γ (IFN-γ), interleukin 4 (IL-4) and β-actin cDNA have previously been published (Varona *et al*. 2005). Real-time PCR was performed using the ABI PRISM 7300 Sequence Detector Machine (PE Applied Biosystem, Foster City, CA, USA) with the commercial kit Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, Paisley, UK). Each sample was analysed in triplicate, and the cycle threshold (Ct) value was obtained with Sequence Detection System Software. All cycle threshold values were normalized to the expression of the housekeeping gene β-actin. For relative quantification of gene expression, the Comparative Threshold Cycle method was used. The relative n-fold change of each target cytokine expression, normalized to the endogenous reference and relative to the control group, is given by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Data analysis

All immunized groups were compared to the non-immunized/challenged group to evaluate the protective efficacy of the vaccine. The influence of the stage-specific antigens and adjuvant type was evaluated by comparing the different antigens (TZ *vs* TZ-BZ) within the same adjuvant group and the different adjuvants (W/O *vs* Al/G) with the same type of antigen, respectively.

Differences in morbidity, mortality, parasite detection and vertical transmission rates were organized in a contingency table. A Chi-squared test or Fisher F-test was performed, and Bonferroni´s adjustment was applied to the *P* value. Post-natal mortality was analysed by the Kaplan-Meier survival method (Bland and Altman, 1998) to estimate the percentage of surviving individuals at each time-point (days PP). To compare the survival curves between groups, the log-rank statistical test was applied (Bland and Altman, 2004). Litter size and serological data were compared using a one-way analysis of variance (one-way ANOVA) test, followed by Tukey's multiple comparison test. All statistical analyses were carried out using Statgraphics Plus v. 5.1 (StatPoint, Inc., Herndon, VA, USA) and GraphPad Prism 5 v. 5.01 (San Diego, CA, USA) software.

Table 2. Litter size, hebdomadal mortality and neonatal mortality of pups in each group

Group	Litter size[a]	Hebdomadal mortality		Median survival time (days)[a]	Neonatal mortality	
		Per pups[b]	Per litters[c]		Per pups[d]	Per litters[e]
1 (W/O-TZ)	6·8 ± 0·8	3/34 (8·8%)	2/5 (40%)	24·7 ± 1·3	16/31 (51·6%)	2/5 (40%)
2 (W/O-TZ-BZ)	5·1 ± 1·8	22/42 (52·4%)	6/8 (75%)	7·3 ± 1·4	18/20 (90%)	7/7 (100%)
3 (W/O-PBS)	4·6 ± 1·1	4/42 (9·5%)	4/9 (44·4%)	19·4 ± 1·0	35/38 (92·1%)	9/9 (100%)
4 (Al/G-TZ)	4·6 ± 1·4	1/37 (2·7%)	1/8 (12·5%)	18·7 ± 1·0	32/36 (88·8%)	8/8 (100%)
5 (Al/G-TZ-BZ)	5·3 ± 1·2	35/53 (66%)	10/10 (100%)	3·1 ± 0·6	18/18 (100%)	10/10 (100%)
6 (Al/G-PBS)	3·8 ± 1·4	5/34 (14·7%)	4/9 (44·4%)	23·4 ± 1·4	19/29 (65·5%)	9/9 (100%)
7 (PBS/TZ)	5·6 ± 1·6	9/79 (11·4%)	4/14 (28·5%)	15·7 ± 0·5	66/70 (94·2%)	14/14 (100%)
8 (PBS/TZ-BZ)	7·0 ± 1·7	13/42 (30·9%)	4/6 (66·6%)	6·2 ± 0·7	29/29 (100%)	6/6 (100%)
9a (non-immunized/ non-challenged)	5·1 ± 1·5	0/37 (0%)	0/8 (0%)	30·0 ± 0	0/37 (0%)	0/8 (0%)
9b (non-immunized/ non-challenged)	5·0 ± 1·6	2/45 (4·4%)	2/10 (20%)	30·0 ± 0	0/43 (0%)	0/10 (0%)
10a (non-immunized/ challenged)	5·2 ± 1·7	8/58 (13·7%)	5/11 (45·5%)	23·2 ± 0·9	42/50 (84%)	11/11 (100%)
10b (non-immunized/ challenged)	5·4 ± 1·7	14/65 (21·5%)	6/12 (50%)	20·0 ± 0·9	43/56 (76·8%)	12/12 (100%)

[a] Average ± S.D.
[b] No. of hebdomadal dead pups/no. of pups born in the group (percentage).
[c] No. of litters with at least 1 stillbirth/no. of litters in the group (percentage).
[d] No. of pups dead from days 2 to 30 PP/no. of pups born alive (percentage).
[e] No. of litters with at least 1 pup dead from days 2 to 30 PP/no. of litters in the group (percentage).

Table 3. Vertical transmission in hebdomadal dead pups and pups born alive as detected by nested PCR

Groups	Hebdomadal dead pups[a]	Pups born alive[b]	Per litter[c]
1 (W/O-TZ)	3/3 (100%)	9/27 (33·3%)	3/5 (60%)
2 (W/O-TZ-BZ)	16/17 (94·1%)	13/14 (92·8%)	7/7 (100%)
3 (W/O-PBS)	2/3 (66·6%)	22/25 (88%)	9/9 (100%)
4 (Al/G-TZ)	ND[d]	31/32 (96·9%)	8/8 (100%)
5 (Al/G-TZ-BZ)	27/28 (96·4%)	9/10 (90%)	10/10 (100%)
6 (Al/G-PBS)	3/4 (70%)	25/27 (92·6%)	9/9 (100%)
7 (PBS/TZ)	6/7 (85·7%)	47/49 (95·9%)	14/14 (100%)
8 (PBS/TZ-BZ)	13/13 (100%)	17/18 (94·4%)	6/6 (100%)
9a (non-immunized/non-challenged)	0/0 (0%)	0/37 (0%)	0/8 (0%)
9b (non-immunized/non-challenged)	0/2 (0%)	0/43 (0%)	0/10 (0%)
10a (non-immunized/challenged)	3/5 (60%)	42/50 (84%)	11/11 (100%)
10b (non-immunized/challenged)	8/11 (72·7%)	43/56 (76·8%)	12/12 (100%)

[a] No. of infected stillborns/no. of stillborns analysed in the group (percentage).
[b] No. of positive pups born alive/no. of analysed pups born alive (percentage).
[c] No. of litters with at least 1 positive pup/no. of analysed litters (percentage).
[d] ND, not determined. No brain samples from hebdomadal dead born pups could be recovered and analysed as a result of cannibalism of the dams observed in all the experimental groups.

RESULTS

Vaccine safety

No systemic side-effects were observed in any animal after immunization. Upon palpation, nodules at the injection site were found in 64·7% to 100% of mice receiving W/O or Al/G, respectively.

Protective efficacy against congenital neosporosis

Different degrees of protection against congenital parasite infection afforded by the different vaccine formulations tested were observed (Tables 2 and 3 and Fig. 1). Dams vaccinated with TZ-BZ (groups 2, 5 and 8) and TZ (group 7) incorporated into PBS transmitted the infection more efficiently to their offspring in comparison with the non-immunized/ challenged group (group 10) and displayed the most severe outcome of congenital neosporosis. A significant increase of hebdomadal mortality (groups 2 and 5 *vs* group 10; $P < 0{\cdot}0001$) and a shorter median survival time of neonates (groups 2, 5, 7 and 8 *vs* group 10; $P < 0{\cdot}05$) were observed in these groups. In contrast, the group vaccinated with TZ and W/O

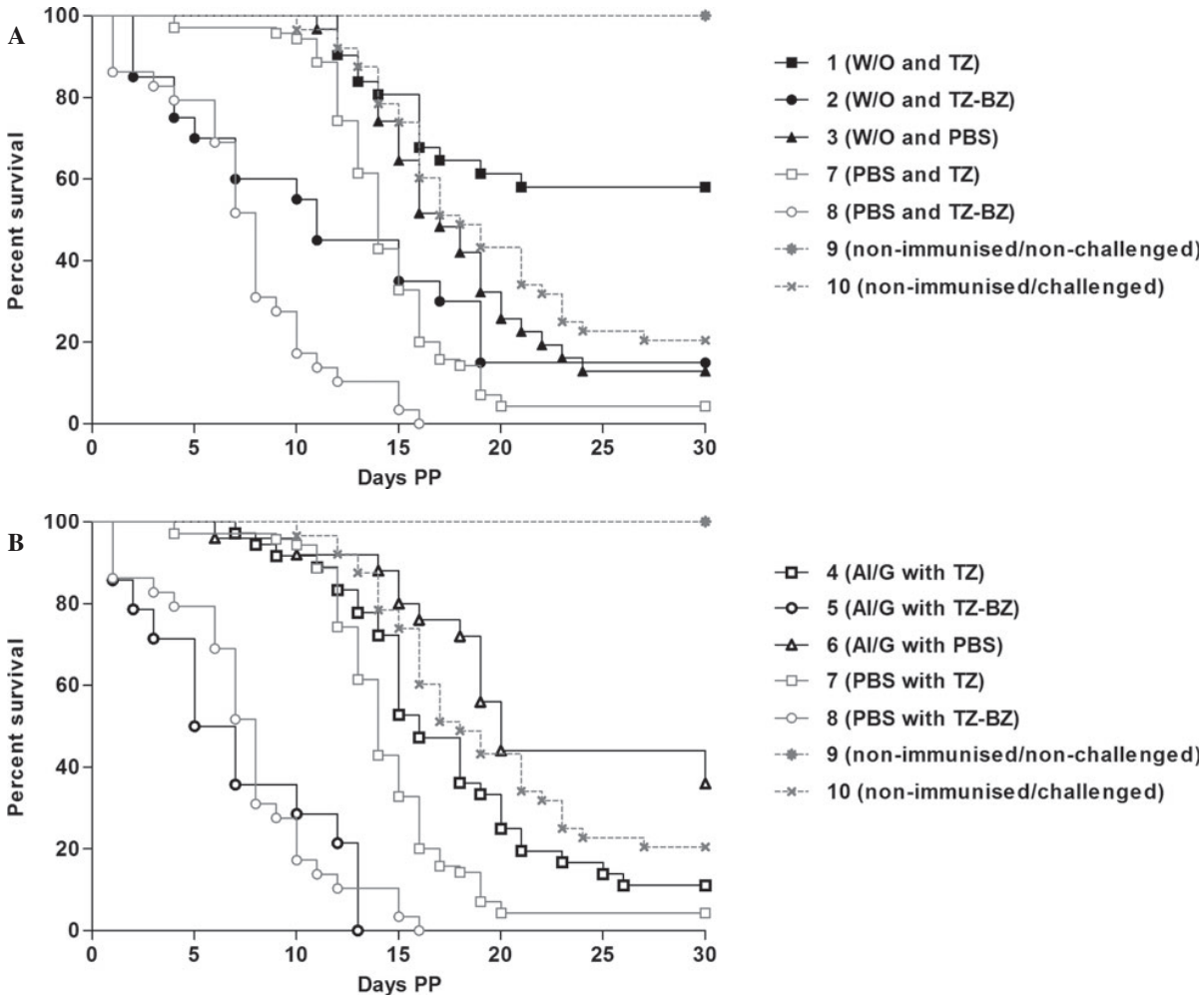

Fig. 1. Kaplan–Meier survival curves for neonates born from dams immunized with adjuvant W/O (panel A) or adjuvant Al/G (panel B). The curves represent the percentage survival as the proportion of all individuals surviving over a period of 30 days PP. Vertical steps downwards correspond to days PP when a death was observed. Symbols indicate censored observations.

(group 1) had a lower mortality rate ($P<0{\cdot}005$), longer median survival time ($P<0{\cdot}005$) and reduced vertical transmission ($P<0{\cdot}0001$) in comparison with the non-immunized/challenged group (group 10).

When the influence of the antigen type was evaluated, higher hebdomadal mortality rates were observed in all groups vaccinated with TZ-BZ and W/O, Al/G or PBS (group 2 *vs* groups 1 and 3; group 5 *vs* groups 4 and 6; group 8 *vs* group 7; $P<0{\cdot}01$). Moreover, pups born alive from dams immunized with TZ-BZ with the Al/G exhibited a shorter median survival time (group 5 *vs* groups 4 and 6; $P<0{\cdot}0001$). Conversely, among the groups vaccinated using the W/O adjuvant, pups from dams given TZ displayed a lower neonatal mortality rate, longer median survival time and lower vertical transmission than pups from groups immunized with TZ-BZ or PBS (group 1 *vs* groups 2 and 3; $P<0{\cdot}0001$).

When the adjuvants were compared, mice vaccinated with TZ-BZ and Al/G showed higher hebdomadal mortality than mice given the same antigen and PBS (group 5 *vs* group 8; $P<0{\cdot}001$). A protective adjuvant-dependent effect was observed in mice vaccinated with TZ and W/O; the pups of these mice that were born alive showed a lower mortality rate, longer median survival time and lower vertical transmission than pups from groups immunized with Al/G or PBS (group 1 *vs* groups 4 and 7; $P<0{\cdot}005$).

Taken together, these results show that immunization with the TZ-BZ antigen exacerbated congenital neosporosis and allowed parasite transmission to offspring during pregnancy. In contrast, immunization with TZ and W/O partially limited vertical transmission of parasite to progeny, preventing offspring infection.

Protective efficacy against cerebral neosporosis

Data on *N. caninum*-related clinical signs, mortality and parasite detection in brain during chronic infection phase are summarized in Table 4. Mice immunized with TZ-BZ (groups 2, 5 and 8) and TZ

Table 4. Morbidity and mortality rates and detection of parasite DNA in adult mice at chronic infection phase

Group	Morbidity [a]	Mortality[b]	Parasite presence[c]
1 (W/O-TZ)	3/16 (18·8%)	2/16 (12·5%)	6/16 (37·5%)
2 (W/O-TZ-BZ)	14/27 (51·9%)	9/27 (33·3%)	24/27 (88·9%)
3 (W/O-PBS)	6/17 (35·3%)	1/17 (5·9%)	10/17 (58·8%)
4 (Al/G-TZ)	11/17 (64·7%)	5/17 (29·4%)	15/17 (88·2%)
5 (Al/G-TZ-BZ)	26/27 (96·3%)	20/27 (74·1%)	26/27 (96·3%)
6 (Al/G-PBS)	3/17 (17·6%)	0/17 (0%)	11/17 (64·7%)
7 (PBS/TZ)	17/17 (100%)	17/17 (100%)	16/17 (94·1%)
8 (PBS/TZ-BZ)	26/26 (100%)	26/26 (100%)	27/27 (100%)
9a (non-immunized/non-challenged)	0/17 (0%)	0/17 (0%)	0/17 (0%)
9b (non-immunized/non-challenged)	0/24 (0%)	0/24 (0%)	0/24 (0%)
10a (non-immunized/challenged)	1/16 (6·25%)	0/16 (0%)	13/16 (81·25%)
10b (non-immunized/challenged)	2/24 (8·3%)	2/24 (8·3%)	21/24 (87·5%)

[a] No. of mice with clinical signs compatible with neosporosis/no. of mice in the group (percentage).
[b] No. of sacrificed mice due to severity of clinical signs/no. of mice in the group (percentage).
[c] No. of nested PCR positive mice/no. of analysed mice in the group (percentage).

(group 7) with PBS exhibited more severe cerebral neosporosis than the non-immunized/challenged group (group 10), showing a higher frequency of clinical signs (groups 2, 5, 8 and 7 *vs* group 10; $P<0{\cdot}001$) and higher mortality rates (groups 5, 8 and 7 *vs* group 10; $P<0{\cdot}0001$). On the contrary, immunization with W/O and TZ reduced the presence of *N. caninum* in the brain compared with mice from the non-immunized/challenged group (groups 1 *vs* group 10; $P<0{\cdot}01$).

The effect of the type of antigen was observed in the group inoculated with Al/G and TZ-BZ, in which clinical signs and mortality were significantly increased compared with those vaccinated with the same adjuvant and TZ or PBS (group 5 *vs* groups 4 and 6; $P<0{\cdot}01$).

When the influence of adjuvant type was evaluated, an increase in morbidity and mortality rates was observed in groups immunized with TZ or TZ-BZ plus PBS or Al/G when compared to groups given the W/O adjuvant (groups 4 and 7 *vs* group 1; groups 5 and 8 *vs* group 2; $P<0{\cdot}01$). Similarly, an adjuvant-dependent increase in mortality rate was observed in mice given TZ or TZ-BZ and PBS with respect to those given the Al/G adjuvant (group 7 *vs* group 4; group 8 *vs* group 5; $P<0{\cdot}01$).

Together, these data show that the TZ-BZ antigen did not limit the establishment of chronic infection in brain and in fact exacerbated cerebral neosporosis. Conversely, immunization with TZ combined with the W/O adjuvant reduced parasite multiplication in the brain during chronic infection.

Immune response prior to challenge

Production of IgG1 was observed in all groups that were immunized with *N. caninum* antigen combined with an adjuvant, but the highest levels were observed after immunization with TZ or TZ-BZ plus W/O (group 1 *vs* groups 7 and 9; group 2 *vs* groups 5, 8 and 9; $P<0{\cdot}0001$) (Fig. 2A). With respect to the IgG2a isotype, the group given TZ-BZ and W/O produced the highest antibody levels (group 2 *vs* groups 5, 8 and 9; $P<0{\cdot}01$).

The anti-rGRA7 IgG response was significantly higher in mice immunized with TZ-BZ and W/O when compared with groups immunized with the same adjuvant, antigen or with non-immunized group (group 2 *vs* groups 1, 3, 5, 8 and 9; $P<0{\cdot}0001$) (Fig. 3A). No *N. caninum*-specific rNcSAG4 and rNcBSR4 IgGs were detected prior to challenge in any immunized groups (Fig. 3B and C).

IFN-γ mRNA expression was induced in groups immunized with *N. caninum* antigen-containing preparations (TZ or TZ-BZ) and was highest in groups immunized with bradyzoite-specific antigens (groups 2, 5 and 8). No up-regulated IL-4 mRNA expression was detected in any immunized group (Fig. 4A).

Immune response after challenge

During the chronic infection phase, mice exhibited a strong anti-*N. caninum* response by producing IgG1 and IgG2a (Fig. 2B). Significant differences in IgG2a production were detected only between some immunized groups when compared with the non-immunized/challenged group (groups 1, 5, 7 and 8 *vs* group 10; $P<0{\cdot}0001$). When the effect of adjuvant or antigen was evaluated, no influence on antibody response was observed.

Regarding the specific antibody response against *N. caninum* recombinant proteins, significantly higher levels of anti-rNcGRA7 IgGs were detected in groups immunized with TZ-BZ (groups 2, 5 and 8 *vs* group 10; $P<0{\cdot}0001$) (Fig. 3A). Interestingly, TZ-BZ-immunized groups produced significantly

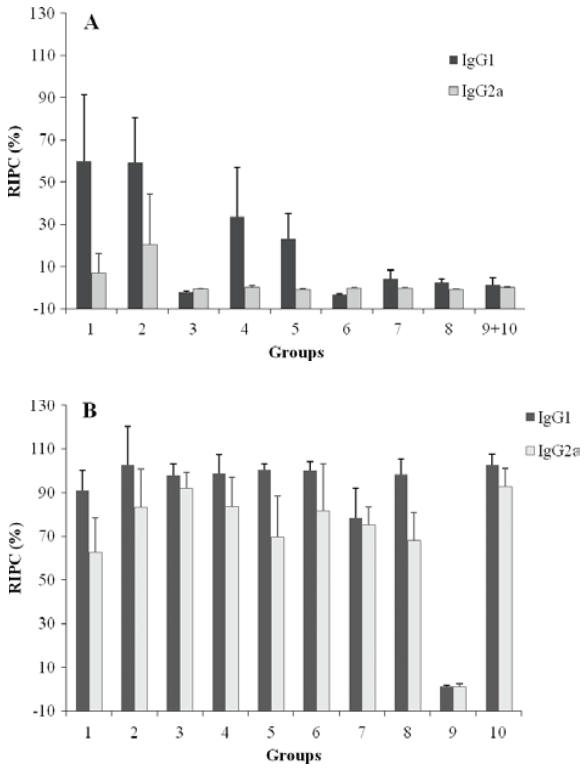

Fig. 2. ELISA anti-*Neospora caninum* IgG1 and IgG2a both from BALB/c mice prior to challenge at day 2 after booster (panel A) and from dams at day 30 PP (panel B). Bars represent the mean relative index percent (RIPC), and error bars indicate the standard deviation for each group. A total number of 5 mice prior to challenge and all of the surviving dams at day 30 PP were included in the analysis. Descriptions of groups 1–10 are summarized in Table 1.

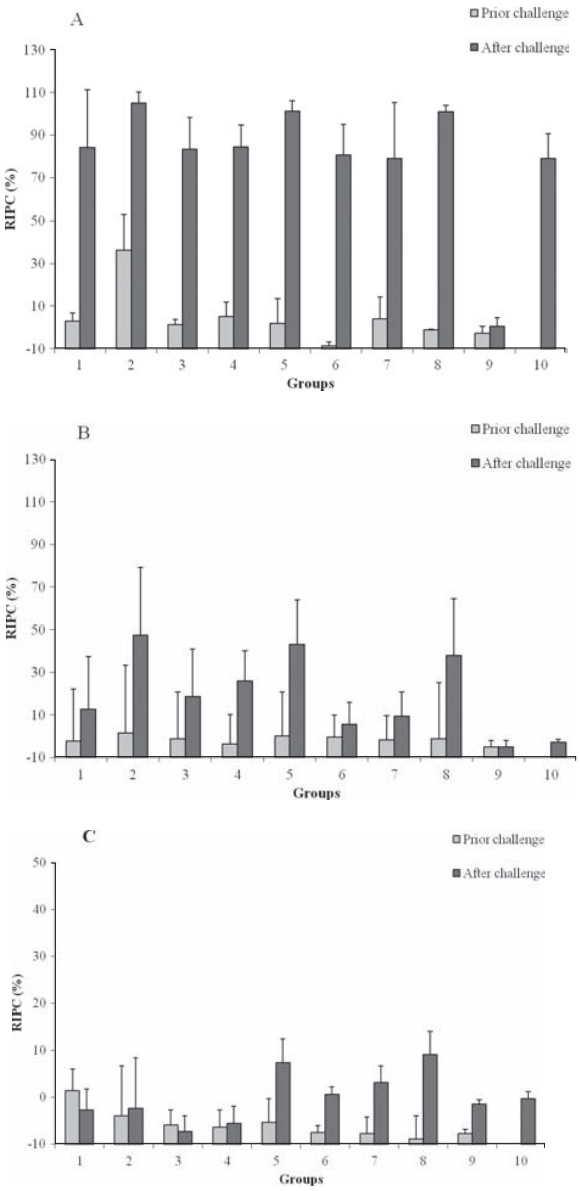

Fig. 3. ELISA results for anti-rNcGRA7 (panel A), anti-rNcSAG4 (panel B) and anti-rNcBSR4 (panel C) antibodies in both BALB/c mice prior to challenge at day 2 after booster and from dams after challenge at day 30 PP. Bars represent the mean relative index percent (RIPC), and error bars indicate the standard deviation for each group. Positive cut-offs were established in GRA7-based ELISA at $\geqslant 7{\cdot}6$ RIPC, in SAG4-based ELISA at $\geqslant 7{\cdot}8$ and in BSR4-based ELISA at $\geqslant 10$ RIPC. Five mice prior to challenge and all of the surviving dams at day 30 PP were included in the analysis. Descriptions of groups 1–10 are summarized in Table 1.

higher levels of antibodies against the recombinant bradyzoite-specific proteins rNcSAG4 (groups 2, 5 and 8 *vs* group 10; $P<0{\cdot}0001$) and rNcBSR4 (groups 5 and 8 *vs* group 10; $P<0{\cdot}0001$) (Fig. 3B).

With regard to the cytokine mRNA expression levels in spleens from dams, groups vaccinated with TZ-BZ (groups 2, 5 and 8) showed the highest IL-4 and IFN-γ transcript levels, with a predominance of IL-4 expression (Fig. 4B).

DISCUSSION

The present study was conducted to evaluate the protective effect of inactivated whole *N. caninum* TZ and TZ-BZ compositions formulated with 2 different adjuvants against congenital and cerebral *N. caninum* infection. The ability of whole TZ antigens to reduce acute parasitaemia and parasite brain infection when combined with Al/G and W/O, respectively, during chronic neosporosis was previously demonstrated in a non-pregnant mouse model (Rojo-Montejo *et al.* 2011). To determine if these formulations conferred protection not only against acute and cerebral neosporosis but also against congenital infection, a well-established pregnant BABL/c mouse model was used (López-Pérez *et al.* 2006, 2008; Regidor-Cerrillo *et al.* 2010). This model is a highly stringent tool for testing the efficacy of vaccine formulations against the transmission of the parasite to progeny (Aguado-Martínez *et al.* 2009*a*) because there is a

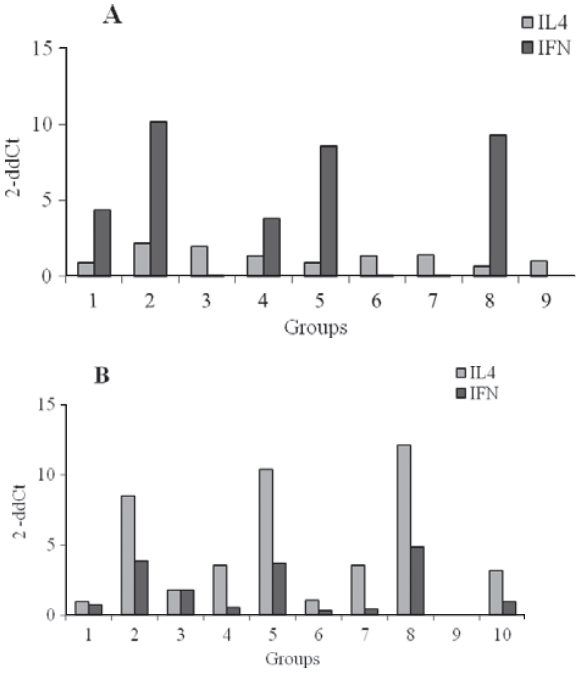

Fig. 4. Cytokine expression in both BALB/c mouse spleen prior to challenge 2 days after booster (A) and after challenge in dams at day 30 PP (B). The results of real-time RT-PCR are given by $-2^{\Delta\Delta Ct}$. The $-2^{\Delta\Delta Ct}$ value for the control is 1. Bars represent the cytokine expression in the pool of each group. Five mice prior to challenge and all the surviving dams at day 30 PP were included in the analysis. Descriptions of groups 1–10 are summarized in Table 1.

high transmission rate of *N. caninum* to the offspring after inoculation of dams at the second trimester of gestation. The results obtained here indicate that different degrees of protection are strongly dependent on the type of antigen and the co-administered adjuvant.

An important feature of cyst-forming protozoan parasites such as *N. caninum* is their ability to establish a chronic infection by converting from rapidly proliferative TZ into BZ that remain hidden from the host immune system contained within tissue cysts. Vaccine preparations composed of antigens from different life-cycle stages may be advantageous to confer protection against the rapid dissemination and invasion of host cells by *N. caninum* TZ and the establishment and maintenance of a chronic infection after conversion of TZ to BZ (Innes and Vermeulen, 2006). To our knowledge, BZ-specific antigens have only been employed as recombinant vaccine candidates against *N. caninum* infection in one instance (Aguado-Martínez *et al*. 2009*a*). In this previous study, we evaluated the induced immune response, safety and efficacy of immunization with rNcSAG4 in a pregnant mouse model. Although no significant protection was found with this vaccine formulation, the decrease in pup mortality indicates that adjusting some aspects of vaccine development, such as ensuring the correct balance of the Th1/Th2 immune response or introducing new vaccine candidates, could lead to more encouraging results. One drawback of using recombinant preparations is that antigens may not retain their native conformation; consequently, their recognition by the immune system may be different, causing a loss of immunogenicity (Cannas *et al*. 2003; Srinivasan *et al*. 2007; Aguado-Martínez *et al*. 2009*a*). In addition, most BZ-specific antigens from the closely related parasite *T. gondii* appear to be poorly or not at all immunogenic during infection, and this condition may be one of the mechanisms by which BZ escape immune surveillance (Di Cristina *et al*. 2004; Kim and Boothroyd, 2005). Due to the complex interaction of the parasite and host, an inactivated whole TZ-BZ mixture composed of a combination of antigens from different life-cycle stages may induce better protective immunity than when the antigens are administered singly, as occurs in our previous study. Recent studies have shown that whole TZ (live, frozen-inactivated or heat-inactivated) preparations achieve a stronger type 1 immune response compared to other *N. caninum* antigen preparations (lysates in the form of total, soluble or insoluble antigen). This response was characterized by an increasing number of IFN-γ-producing NK cells and the elicited IL-12 and TNF-α production by bone marrow-derived DCs and high levels of IFN-γ by spleen cells (Klevar *et al*. 2007; Strohbusch *et al*. 2009; Feng *et al*. 2010). Therefore, in the present study, we compared the effect of immunization with 2 different inactivated whole antigen preparations (TZ and TZ-BZ). Zoites for vaccine formulations were obtained from the Nc-Spain7 isolate of *N. caninum* (Regidor-Cerrillo *et al*. 2008), which transforms from TZ into BZ after stress by the SNP agent in cell culture. Because the conversion to BZ is a process that can be triggered by immune-derived stress, this *in vitro* procedure mimics early events of the TZ-BZ switch, in which mixed parasitophorous vacuoles composed of both TZ and BZ are obtained on day 7 after stress induction. Recently, we identified differentially expressed proteins during the tachyzoite-bradyzoite stage conversion, which are likely involved in early events of bradyzoite development, using the same *in vitro* conversion procedure and DIGE technology (Marugán-Hernández *et al*. 2010). Therefore, our initial hypothesis was that vaccination of mice with a TZ-BZ mixture, containing bradyzoite proteins upregulated early during the conversion of TZ to BZ, may induce an effective immune response against initial steps of bradyzoite cyst formation.

Surprisingly, TZ-BZ-vaccinated groups presented the shortest median survival time of pups and the highest morbidity and mortality rates in adult mice, indicating exacerbated congenital and cerebral neosporosis. These findings were associated with higher antibody levels against the *N. caninum* stage-specific

proteins rNcGRA7, rNcSAG4 and rNcBSR4 after challenge. As mentioned above, the immunogenic dense granule protein NcGRA7 is highly involved in active replication of the parasite and host cell invasion (Augustine *et al*. 1999; Cho *et al*. 2005). The high antibody production against rNcGRA7 points to widespread tachyzoite proliferation that may explain the fatal outcome of infection. On the other hand, the magnitude of antibody levels against both bradyzoite-specific proteins rNcSAG4 and rNcBSR4 may indicate an antigenic re-stimulation as a consequence of the conversion to BZ by some parasites after challenge, probably to escape detection by the host immune system and to establish a persistent infection. In this study, immunization with TZ-BZ induced a polarized type 1 immune response that was associated with a dramatic dissemination of parasites throughout the organism, where they crossed biological barriers such as the placenta or the blood-brain barrier and actively invaded fetal tissues and brain from adult mice. These results are in contrast with previous conventions describing the protective role of the Th1 immune response against *N. caninum* by parasite clearance via IFN-γ production (Khan *et al*. 1997; Nishikawa *et al*. 2001*a*; Yamane *et al*. 2000). The immunological mechanisms by which TZ/BZ immunization led to the exacerbation of the disease are not clear. The immune-derived stress could favour the rapid dissemination of TZ to immune-privileged tissues, such as the brain, to evade the immune response, potentially allowing uncontrolled TZ invasion and replication to occur. The detection of the highest levels of antibody against bradyzoite-specific proteins may indicate that, once in the brain, some parasites began the conversion process and that immunization was not able to block the first steps of the conversion from TZ to BZ as we initially hypothesized. Because proper techniques to detect tissue cysts in the brain were not used, further studies are required to determine whether vaccination with TZ-BZ antigens is able to impair both cyst formation and successful establishment of a chronic infection. Alternatively, inflammatory cytokine-mediated immunopathological changes may also contribute to disease severity. Dysregulation of the balance between Th1 and Th2 responses by the hyper-production of Th1-related cytokines such as IFN-γ was also associated with a failure in the protection against toxoplasmosis and neosporosis in different vaccine assays (Kim and Boothroyd, 2005; Aguado-Martínez *et al*. 2009*a*; Ribeiro *et al*. 2009). Interestingly, a different pattern of cytokine expression was observed during chronic infection in the spleens of mice immunized with TZ-BZ, where IL-4 expression was up-regulated. For other intracellular protozoa such as *Leishmania*, it has been suggested that parasite load might affect the type of immune response developed. High parasite load would favour a Th2 response, which acts directly to down-regulate Th1 cells (Hondowicz and Scott, 2002). The more rapid dissemination and uncontrolled parasite multiplication within host tissues in TZ-BZ-vaccinated groups may induce a progressive impairment of immune functions, dysregulating the delicate balance between infection control and host survival and finally resulting in an exacerbated neosporosis.

In accordance with previous results (Rojo-Montejo *et al*. 2011), vaccination with inactivated whole TZ and the W/O emulsion induced partial protection to congenital infection by significantly reducing neonatal mortality (from 84% to 51·6%) and vertical transmission (84% to 33·3%) and conferred complete protection in 2 out of 5 (40%) litters. A strong humoral immune response with predominant IgG1 production and a cellular response dominated by IFN-γ cytokine expression were produced prior to challenge with W/O and TZ. This result suggests mixed Th1/Th2 responses that may be beneficial to limit dissemination and protect offspring against congenital infection of *N. caninum*. Previously, a study in congenital neosporosis showed that protection against vertical transmission in mice immunized with different recombinant vaccinia viruses was related to the cellular immune response and high IgG1 antibody levels. These results suggest a role for a high level of IgG1 production in the clearance of *N. caninum* at early stage of infection and the role of a T cell response in later stage of infection (Nishikawa *et al*. 2001*b*).

Immunization of mice with TZ and Al/G failed to confer protection against transplacental transmission and cerebral infection of *N. caninum,* as we have observed previously (Rojo-Montejo *et al*. 2011). A slight improvement in vaccine formulation was detected because no exacerbated neosporosis was observed in mice immunized with TZ in the vaccine containing Al/G. However, the immune response induced by immunization with TZ and Al/G was clearly insufficient to generate significant protection against cerebral and congenital neosporosis.

Although the focus of the present study was the evaluation of safety and efficacy, antibody and cytokine responses were also measured. Interestingly, differences in cytokine expression levels depending on the type of antigen inoculated were observed between immunized groups, as indicated above. While the cytokine mRNA expression data indicate a strong trend, all cytokine measurements were performed using pooled spleen samples, and consequently, the data could not be statistically analysed. Thus, the data should be interpreted with caution.

In summary, the present study highlights the critical role of stage-specific antigens and different adjuvants in the development of effective inactivated vaccines for the prevention of *N. caninum* infection. Immunization with TZ-BZ considerably affected the immune response generated against *N. caninum*

infection, after which the most severe congenital and cerebral neosporosis were detected, probably due to an inadequate balance of immune response. On the contrary, partial protection including reduced vertical transmission and neonatal mortality was observed in the group given inactivated whole *N. caninum* TZ combined with the W/O adjuvant. Although the present study showed discouraging results on the use of bradyzoite antigens as vaccine candidates, immunization against different stages of *N. caninum* is still a promising approach in vaccine developments. Further studies are required to identify the bradyzoite-specific proteins that induced protection against chronic infection and reactivation of the parasite by blocking the TZ-to-BZ conversion and vice versa.

ACKNOWLEDGEMENTS

We thank Louis M. Weiss (Albert Einstein College of Medicine, New York, USA) for the polyclonal rabbit anti-recombinant BAG1 protein from *T. gondii* serum, Andrew Hemphill (Institute for Parasitology, University of Berne, Switzerland) for providing the monoclonal mouse anti-recombinant SAG1 (*N. caninum*) and Diana Williams (Liverpool School of tropical Medicine, Liverpool, UK) for kindly providing the Nc-Liverpool isolate. We would also like to thank Vanesa Navarro Lozano for her excellent technical assistance. The Nc-Spain7 isolate has been patented by the SALUVET group (PCT/ES08/00492; US2010/0255577).

FINANCIAL SUPPORT

This work was supported by the PETRI 95-0777.OP project from the Spanish Government and HIPRA.

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Capítulo IV

Veterinary Parasitology 159 (2009) 7–16

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Isolation and characterization of a bovine isolate of *Neospora caninum* with low virulence

Silvia Rojo-Montejo [a], Esther Collantes-Fernández [a], Javier Regidor-Cerrillo [a], Gema Álvarez-García [a], Virginia Marugan-Hernández [a], Susana Pedraza-Díaz [a], Javier Blanco-Murcia [a], Antonio Prenafeta [b], Luis Miguel Ortega-Mora [a,*]

[a] *SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain*
[b] *Hipra Laboratories, S.A. Avda. de La Selva s/n, 17170 Amer, Girona, Spain*

ARTICLE INFO

Article history:
Received 27 June 2008
Received in revised form 6 September 2008
Accepted 3 October 2008

Keywords:
Neospora caninum
Low virulence isolate
Biological characterization

ABSTRACT

Neospora caninum tachyzoites were isolated from the brain of an asymptomatic naturally infected calf with precolostral-specific antibodies. The new isolate, named Nc-Spain 1H, was identified as a member of the *N. caninum* species based on its internal transcribed spacer 1 (ITS-1) sequence and was genetically characterized using microsatellite markers. Multilocus analysis showed that Nc-Spain 1H was genetically different from other *N. caninum* isolates. We compared the *in vitro* tachyzoite yield and viability rate of the Nc-Spain 1H and Nc-1 isolates in a plaque assay. The lower tachyzoite yields displayed by Nc-Spain 1H were complemented with a significantly lower viability rate. Moreover, in an *in vitro* tachyzoite–bradyzoite stage conversion assay, the percentage of Nc-Spain 1H bradyzoite conversion was similar to that of the cystogenic isolate Nc-Liv, with the exception that Nc-Spain 1H produced only intermediate bradyzoites. The pathogenicity of Nc-Spain 1H was examined in BALB/c mice, and the results demonstrated that Nc-Spain 1H failed to induce clinical signs or mortality and that no parasite DNA was detected in the brain during the chronic stage of infection. In a pregnant mouse model, Nc-1 infection resulted in high transplacental transmission, leading to a high neonatal mortality rate over time. In contrast, the offspring survival rate from Nc-Spain 1H-infected dams was almost 100%, and *N. caninum* DNA was detected in only one pup. These data show that Nc-Spain 1H appears to be a low virulence isolate and may be a suitable candidate for live vaccine development.

1. Introduction

Neospora caninum is a cyst-forming coccidian parasite that is closely related to *Toxoplasma gondii* and has been recognized worldwide as a cause of neuromuscular disease in dogs and abortion in cattle (Dubey et al., 2006). The predominant route of transmission of the parasite in cattle is considered to be transplacental and may play a significant role in maintenance of the infection within a herd. Postnatal transmission, due to ingestion of sporulated *N. caninum* oocysts, can contribute to the sustainability of *N. caninum* infection, and it has been associated with abortion outbreaks (Wouda, 2007). Fetuses may die *in utero*, be resorbed, mummified, autolyzed, stillborn, born alive with clinical signs, or born clinically normal but with chronic infection (Dubey and Schares, 2006). Advances concerning the *N. caninum* life cycle have proven that dogs and coyotes are both intermediate and definitive hosts, whereas cattle and other mammals are natural intermediate hosts (Dubey et al., 2006). *N. caninum* has been

* Corresponding author. Tel.: +34 91 3944069; fax: +34 91 3944098.
E-mail address: luis.ortega@vet.ucm.es (L.M. Ortega-Mora).

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doi:10.1016/j.vetpar.2008.10.009

isolated from dogs, cattle, sheep, water buffaloes, and white tailed deer (Dubey et al., 2006, 2007).

Various authors have described the existence of the intra-specific variability of *N. caninum*, since differences in *in vitro* growth and virulence in mouse models have been observed among different isolates (Atkinson et al., 1999; Schock et al., 2001; Quinn et al., 2002; Miller et al., 2002; Pérez-Zaballos et al., 2005; Collantes-Fernández et al., 2006). In fact, the Nc-Nowra isolate, which was obtained from a congenitally infected calf, demonstrated low virulence in mice (Miller et al., 2002). Such intra-specific diversity may be associated with variation in the clinical presentation of disease. With regard to variability in the clinical presentation, little is known about differences among *N. caninum* isolates from symptomatic or asymptomatic animals, since a limited number of isolates have been described and most characterized isolates were obtained from animals with clinical signs. In addition, analyses of the genetic diversity of *N. caninum* are limited. Random Amplified Polymorphic DNA-PCR (RAPD-PCR) and sequence analysis of rDNA internal transcribed spacer 1 (ITS-1) regions have been used to demonstrate intra-species diversity (Atkinson et al., 1999; Davison et al., 1999; Spencer et al., 2000; Schock et al., 2001; Gondim et al., 2004), but these techniques utilize genomic regions that are insufficiently polymorphic to differentiate between *N. caninum* isolates. The microsatellite technique has recently been used to demonstrate significant genetic diversity within *N. caninum*, enabling detailed studies of the genetic complexity of *N. caninum* infections (Regidor-Cerrillo et al., 2006).

This report describes the isolation and characterization of a new isolate from an asymptomatic, naturally infected calf that was designated Nc-Spain 1H and showed low virulence in mice. Genetic characterization was carried out by ITS-1 sequencing and microsatellite marker analysis, and antigenic features were determined by western blot. The *in vitro* behavior of the Nc-Spain 1H isolate was studied by evaluating tachyzoite yield and viability, as well as bradyzoite development in cell cultures. Finally, pathogenicity in a BALB/c mouse model was also investigated.

2. Materials and methods

2.1. Nc-Spain 1H isolation procedure

2.1.1. Case report

The parasite was isolated from a clinically healthy Holstein–Friesian 2-week-old female calf with a precolostral *N. caninum* antibody titer of 1:500, as estimated by an indirect fluorescent antibody test (Álvarez-García et al., 2002). The dam of the calf was *N. caninum*-positive and came from a dairy herd in Madrid (Spain) with high intra-herd *N. caninum* seroprevalence. The calf was sacrificed, and the brain was analyzed to confirm *N. caninum* infection. A specific ITS-1 nested-PCR test (Buxton et al., 1998) amplified *N. caninum* DNA, and histological examination revealed lesions compatible with protozoan infection in the brain (Barr et al., 1991), characterized by isolated gliosis foci and perivascular cuffing in all bovine sections analyzed.

2.1.2. Bioassay in nude mice and cell culture

PCR-positive calf brain portions were homogenized, filtered in sterile gauze, and centrifuged at 1350 × *g* for 15 min. The sediment was resuspended in PBS with antibiotics (2000 IU/ml penicillin G and 200 μg/ml of streptomycin) (Gibco) and four 4-week-old female nude mice (Charles River, Barcelona, Spain) were inoculated intraperitoneally (i.p.) with homogenized calf brain tissue equivalent to 5 g. The mice were examined daily, and all animals showed clinical neosporosis at 32–40 days post-inoculation (p.i.). Apathy was the first sign of disease, followed by wasting and inactivity. In addition, a nested-PCR test was performed on murine brain samples to confirm the presence of *N. caninum* DNA, and all samples tested were positive.

For *in vitro* cultivation, the mouse peritoneal cavity was flushed, and peritoneal washes were inoculated onto a 24-h cell monolayer culture of MARC-145 cells. The cell cultures were subsequently passaged onto a fresh monolayer cell culture every 4–7 days. *Neospora*-like tachyzoites were observed 24 days after the fourth passage in cell culture. This new isolate was called Nc-Spain 1H. A sample of the Nc-Spain 1H isolate was deposited in the *Culture Collection of Algae and Protozoa* (Oban, Scotland, UK) under the CCAP Accession Number 2051/2.

2.2. Genetic characterization of the Nc-Spain 1H isolate

2.2.1. ITS-1 sequencing

NN1 and NN2 primers designed by Buxton et al. (1998) were used to amplify and sequence the ITS-1 region from Nc-Spain 1H, Nc-Liv (Barber et al., 1995), and Nc-SweB1 (Stenlund et al., 1997) isolates. Parasites were purified from the cell monolayer by rupture with a 25-gauge needle, followed by centrifugation (1350 × *g*, 4 °C, 15 min). The pellet was resuspended in cold PBS, washed once by centrifugation, and resuspended in 5 ml of PBS, and parasites were separated from host cells on PD-10 columns (Amersham Biosciences), as described previously (Hemphill, 1996). DNA was extracted from 10^8 tachyzoites as described below. Amplified products were visualized under UV light in a 1.5% agarose/ethidium bromide gel, purified using the Geneclean Turbo kit (Q-BIOgene, Carlsbad, USA) according the manufacturer's instructions, and then directly sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) and a 3730 DNA analyzer (Applied Biosystems) at the Unidad Genómica del Parque Científico de Madrid. PCR products were sequenced twice in both directions. Sequences were analyzed using the BioEdit Sequence Alignment Editor v.7.0.1 (Copyright© 1997–2004 Tom Hall, Ibis Therapeutics, Carlsbad CA 92008, USA).

2.2.2. Microsatellite analysis

Fifty nanograms of parasite DNA were used to amplify 13 microsatellites, as previously described (Regidor-Cerrillo et al., 2006). PCR products were visualized under UV light in a 2.5% NuSieve 3:1-agarose/ethidium bromide gel (Nu-Sieve 3:1, Cambrex BioScience, USA), purified using the Exo-SAP IT kit (USB, USA) according the

manufacturer's instructions, and then directly sequenced and analyzed as described above.

2.3. Western blot analysis

Western blot analysis was performed under reducing conditions according to a previously described procedure (Álvarez-García et al., 2002; Chávez-Velásquez et al., 2005) with 2×10^7 tachyzoites/gel of purified Nc-Spain 1H and Nc-1 isolates (Dubey et al., 1988). Murine and bovine sera were tested at dilutions of 1:50 and 1:20, respectively. As secondary antibodies, a rabbit polyclonal anti-mouse IgG (whole molecule) (1:500) (Sigma Chemical Co.) and a mouse monoclonal anti-bovine IgG1 and IgG2 antibody (1:400) (Hipra Laboratories S.A.; Gerona, Spain) were used. Murine plasma pools were made by mixing an equal volume of plasma per mouse obtained on day 32 p.i. from BALB/c mice infected with 10^6 and 10^7 Nc-Spain 1H tachyzoites and mice infected with 10^6 Nc-1 tachyzoites from previous studies (Collantes-Fernández et al., 2006). A pool of plasma from mice injected with PBS was also included as a negative control. Cattle serum samples included precolostral serum from the naturally infected calf from which Nc-Spain 1H was isolated, precolostral positive sera from several field cases of congenitally infected calves, and a precolostral negative serum sample as a control.

2.4. "In vitro" behavior of the Nc-Spain 1H isolate

2.4.1. Tachyzoite yield and viability assays

For both the tachyzoite yield and viability assays, the reference Nc-1 isolate obtained from a dog with clinical signs (Dubey et al., 1988) was employed as a control. For this, Nc-1 tachyzoites were propagated under new culture conditions using MARC-145 cells. This shift from Vero cells to a new cell line was expected to homogenize cell passage in Nc-1 (Pérez-Zaballos et al., 2005). Prior to the experiment, Nc-1 and Nc-Spain 1H tachyzoites were maintained *in vitro* by continuous passage in MARC-145 cell monolayers as previously described (Pérez-Zaballos et al., 2005) to ensure healthy actively replicating parasites. The experiment was carried out using similar parasite passage numbers in MARC-145 cells for Nc-1 (passage no. 22) and Nc-Spain 1H (passage no. 14). First, the tachyzoite yield was determined in MARC-145 monolayers grown in 25 cm^2 tissue culture flasks inoculated with Nc-1 and Nc-Spain 1H isolates. The number of tachyzoites and, consequently, the infection dose were determined by Trypan blue exclusion followed by counting in a Neubauer chamber. An optimized multiplicity of infection (MOI) was used for each isolate, with the aim of estimating the tachyzoite yield of both isolates in optimal conditions. Thus, the differences observed between parasite strains are not simply a consequence of the infection efficiency. As a result, we obtained a maximum number of infected cells and parasite vacuoles 3.5 days post-infection, and then the number of both total and viable tachyzoites recovered was estimated for each isolate by Trypan blue exclusion. The experiment was performed twice, with individual samples assessed in triplicate. Second, the viability assay was carried out as a plaque assay. Thus, for each isolate, 100 tachyzoites were inoculated into a 24-well plate containing MARC-145 monolayers (six wells per isolate) grown on 10 mm coverslips. At day 5 p.i., the number of plaque forming tachyzoites per well were determined by counting the lysed areas utilizing a direct immunofluorescent test with rabbit antiserum developed against *N. caninum* (Nc-1 isolate), as described previously (Risco-Castillo et al., 2004).

2.4.2. Tachyzoite–bradyzoite stage conversion

This study determined the tachyzoite–bradyzoite conversion rate of Nc-Spain 1H compared with the cystogenic Nc-Liv isolate. We compared Nc-Spain 1H only with Nc-Liv because the Nc-1 tachyzoite–bradyzoite conversion rate is very low in cell culture (data not shown). The tachyzoite–bradyzoite conversion rate was observed by culturing infected MARC-145 cells with medium containing 70 μM SNP for up to 6.5 days. Control wells were maintained with medium at pH 7.2 for 3–4 days (Risco-Castillo et al., 2004). Cell cultures were inspected, and the medium was changed daily. Nc-Liv tachyzoites were maintained at a 0.5:1 host-parasite ratio, whereas the Nc-Spain 1H isolate was assayed at an MOI of 2.5. At 3, 5, and 6.5 days after stress, the tachyzoite–bradyzoite conversion rate was assessed using a double immunofluorescence assay. Identification of tachyzoite and bradyzoite development can be discerned by labeling with antibodies directed against the immunodominant *N. caninum* tachyzoite surface antigen NcSAG1 (Fuchs et al., 1998) and antiserum against *T. gondii* bradyzoite antigen 1 (BAG1), which exhibits cross-reactivity with *N. caninum* bradyzoites (McAllister et al., 1996). This technique has been broadly utilized in previous *N. caninum* tachyzoite–bradyzoite conversion studies (Weiss et al., 1999; Vonlaufen et al., 2002; Risco-Castillo et al., 2004). Thus, coverslips were labeled with a monoclonal mouse antibody directed against the tachyzoite surface antigen NcSAG1 (αSAG1) (1:2000) and a polyclonal rabbit antiserum raised against the intracytoplasmic bradyzoite antigen BAG1 (αBAG1) (1:100) (Risco-Castillo et al., 2004) by incubation at room temperature (RT) for 1 h. The incubation was followed by two brief washes and three 5 min washes in PBS containing 0.1% BSA, followed by incubation with 0.1 mg/ml 49,6-diamidin-2-phenylindole (Sigma Chemical Co., St. Louis, Mo, USA) and the appropriate conjugates (Molecular Probes) (RT, 1 h). The coverslips were washed as previously described, including a final wash in distilled water. Finally, the coverslips were overlaid with 40% glycerol and 2.5% 1,4-diazabicyclo[2,2,2]octane (Sigma Chemical Co.) in PBS. Antibody binding was observed with a 100× oil-immersion objective on a fluorescence-inverted microscope (Nikon Eclipse TE200). The experiment was performed twice, and individual samples were evaluated in triplicate. The tachyzoite–bradyzoite conversion rate was calculated by random counting of 10 fields per coverslip and comparing the percentage of BAG1-positive parasitophorous vacuoles (PV) containing either pure or intermediate bradyzoites versus total PV.

2.5. Pathogenicity studies of the Nc-Spain 1H isolate in BALB/c mice

2.5.1. Experiment 1

Transmission of the parasite during pregnancy was examined in a pregnant BALB/c mouse model (López-Pérez et al., 2006, 2008), and differences between the Nc-Spain 1H and Nc-1 isolates were studied. Eight-week-old female mice (Harlan Interfauna Ibérica, Barcelona, Spain) were mated for four nights following synchronization of ovulation using the Whitten effect (Whitten, 1957). The mice were inoculated subcutaneously with 2×10^6 tachyzoites of Nc-Spain 1H, Nc-1, or PBS at mid-gestation (6–10 days of pregnancy). Gestation was evaluated by determining the weight of the mice on day 18 after the first night mated. Pregnant mice ($\geq$25 g) were housed individually and allowed to carry their pregnancy to term. Neonates and dams were sacrificed with CO_2 gas on day 30 post-partum (P.P.). Litter size was defined as the number of pups delivered per dam. Body weight was determined every 2 days from 14 to 20 days P.P. to avoid excessive handling of the pups and their consequent rejection by the dams. Stillbirth was evaluated as the number of full-term dead pups at the time of birth. Neonatal mortality was considered as the number of dead pups from birth to 30 days P.P. Clinically affected mice were humanely sacrificed by CO_2 inhalation. The vertical transmission rate was determined by evaluating the presence of the parasite in the lung or brain of neonates by nested-PCR. Brain and lung samples were collected from sacrificed neonates and frozen at −80 °C for DNA extraction. Blood samples from dams were collected by cardiac puncture, and the recovered sera were aliquoted and cryopreserved at −80 °C for ELISA. Brains from dams were also removed and frozen (−80 °C) until used for PCR amplification. The remaining mice that did not result in pregnancy were used to evaluate chronic *N. caninum* infection (Collantes-Fernández et al., 2006). They were sacrificed with CO_2 gas 30 days p.i., and sera and brains were recovered for ELISA and PCR testing, respectively, as described above.

2.5.2. Experiment 2

An inoculum dose titration in the BALB/c mouse model (Collantes-Fernández et al., 2006) was performed in order to comprehensively investigate Nc-Spain 1H pathogenicity. Six-week-old female mice (Harlan Interfauna Ibérica, Barcelona, Spain) were inoculated with 10^5, 10^6, or 10^7 Nc-Spain 1H tachyzoites by i.p. injection of a final volume of 200 μl/mouse, as previously described (Collantes-Fernández et al., 2006). A control group of BALB/c mice was i.p. inoculated with 200 μl of PBS. Three random animals from each group were sacrificed with CO_2 gas on days 1, 2, 4, 8, 16, and 32 p.i., excluding the group inoculated with 10^7 tachyzoites, for which four mice were killed on days 1, 2, and 4. The day of infection was referred to as day 0, and animals were monitored daily. Blood samples (300–500 μl) were collected in EDTA tubes by cardiac puncture and centrifuged ($2000 \times g$, 10 min), and the plasma was recovered, aliquoted, and cryopreserved at −80 °C for ELISA and western blot analysis. Peripheral blood cells were stored at 4 °C for no longer than 24 h for DNA extraction. Target organs (lungs and brains) were recovered under stringent aseptic conditions to avoid cross-contamination and then frozen (−80 °C) until analyzed by *N. caninum* PCR amplification.

2.6. DNA extraction and ITS-1 nested-PCR

The GenomicPrep cell and tissue DNA isolation kit (Amersham Biosciences, Uppsala, Sweden) was used to extract DNA from *N. caninum* tachyzoites and 10–20 mg of each host tissue, and the GenomicPrep blood DNA isolation kit (Amersham Biosciences, Uppsala, Sweden) was used to extract DNA from blood samples according to the manufacturer's instructions.

Nested-PCR of the ITS-1 region of *N. caninum* was carried out with four oligonucleotides, as described by Buxton et al. (1998). The secondary amplification product was visualized by 1.8% agarose gel electrophoresis and ethidium bromide staining. To avoid false positive reactions, DNA extraction, PCR sample preparation, and electrophoresis were performed in separate rooms employing different sets of instruments, aerosol barrier tips, and disposable gloves. Moreover, negative control samples were included in each set of DNA extractions and PCR reactions.

2.7. Humoral immune responses to experimental murine infections

N. caninum-specific serum isotypes, IgG2a and IgG1, were determined by ELISA using a soluble *N. caninum* tachyzoite antigen. Briefly, 96-well plates were coated with soluble *N. caninum* tachyzoite antigen (0.5 μg in 100 μl/well), and diluted murine serum samples (1:100) and anti-mouse IgG1 or IgG2a antibody (1:5000; Southern Biotechnology, Birmingham, AL, USA) were used as described previously (Collantes-Fernández et al., 2006). The serum isotype balance was evaluated via the IgG1/IgG2a ratio.

2.8. Data analysis

In *in vitro* assays, differences in tachyzoite yield and viability between isolates were evaluated using the non-parametric Mann–Whitney *U*-test, and the percentages of PV expressing BAG1 over time were compared by the Student's *t*-test. In the pathogenicity studies, a one-way ANOVA followed by Duncan's Multiple Range test was employed to compare serum anti-*N. caninum* antibody values, litter size, and neonate body weight. Differences in PCR detectability and vertical transmission were analyzed by the Chi-square and Fisher *F*-tests. Neonatal mortality was analyzed by the Kaplan–Meier survival method (Bland and Altman, 1998) for estimation of the portion of surviving individuals 30 days P.P. The log-rank statistical test was applied to compare the survival curves (Bland and Altman, 2004). Statistical analysis was carried out using SAS 8.02 (SAS Institute, Cary, NC, USA) and GraphPad Prism 4 v.4.03 (San Diego, CA, USA).

3. Results

3.1. Genetic and immunogenic characterization

No differences were detected among the ITS-1 sequences obtained from the Nc-Spain 1H, Nc-Liv, and Nc-SweB1 isolates in this study when they were compared with those previously deposited in the GenBank database corresponding to Nc-Liv (accession number AY259038) and Nc-SweB1 (accession number AY259038), with the possible exception of one ambiguous base (K) discovered in the Nc-Spain 1H and Nc-Liv sequences at position 373. The nucleotide sequences of the Nc-Spain 1H, Nc-Liv, and Nc-SweB1 isolates obtained in this study were deposited in the GenBank database under accession numbers EU564165, EU564166, and EU564167, respectively.

In addition, alleles for each of 13 microsatellites used in a previous study (Regidor-Cerrillo et al., 2006) were amplified and sequenced from the Nc-Spain 1H isolate. Most alleles detected in this isolate were previously identified in an analysis of nine isolates by Regidor-Cerrillo et al. (2006), except for microsatellite markers MS3 and MS6A, for which new alleles with 11 and 18 dinucleotide repeat units, respectively, were described (Table 1). Thus, multilocus analysis showed a unique profile for the Nc-Spain 1H isolate. The nucleotide sequences of the microsatellites analyzed in this study have been deposited in the GenBank database under the accession numbers specified in Table 1.

The antigenic profiles detected by western blotting were the same for plasma samples collected from Nc-Spain 1H- and Nc-1-inoculated mice. Precolostral positive calf sera also recognized similar profiles in both cases. The pattern of antigen recognition corresponded to the *N. caninum* immunodominant antigens previously described (Álvarez-García et al., 2002), with molecular weights of 17–18, 34–35, 37, and 60–62 kDa (data not shown). No antigens were detected when negative sera were analyzed.

3.2. "In vitro" behavior of the Nc-Spain 1H isolate

The Nc-Spain 1H isolate, when inoculated onto a cell monolayer, showed cytopathogenic effects similar to the Nc-1 isolate, which is associated with the rupture of infected cells. However, the Nc-1 isolate destroyed 80% of the cell monolayer, and many tachyzoites were observed in lysed areas, whereas only 20% of the Nc-Spain 1H-infected monolayers were disrupted 3.5 days post-infection, subsequently revealing significantly different tachyzoite yields ($P < 0.01$, Mann–Whitney *U*-test) (Fig. 1A). Nevertheless, clusters of Nc-Spain 1H tachyzoites were observed outside the cells. The lower parasite tachyzoite yield displayed by Nc-Spain 1H was complemented by a significantly lower viability rate when compared with the Nc-1 isolate in a plaque assay ($P < 0.01$, Mann–Whitney *U*-test). Five days post-infection, 17.3% and 36.5% of the plaque-forming tachyzoites were observed in wells inoculated with the Nc-Spain 1H and Nc-1 isolate, respectively.

The tachyzoite–bradyzoite conversion rate was estimated from 3 to 6.5 days after stress for both the Nc-Spain

Table 1
Assignment of alleles and the allelic profile obtained for the Nc-Spain 1H isolate.

Microsatellite loci	MS1A	MS1B	MS2	MS3	MS4	MS5	MS6A	MS6B	MS7	MS8	MS10	MS12	MS21
Allele no.	1	1	3	5	4	1	6	2	3	4	4	2	1
Repeat length	$(TA)_{60}$	$(AT)_{12}$	$(AT)_{23}$	$(AT)_{11}$	$(AT)_{18}$	$(TA)_{11}$	$(TA)_{18}$	$(AT)_{12}$	$(TA)_{14}$	$(AT)_{16}$	$(ACT)_6(AGA)_{21}(TGA)_{10}$	$(GT)_{16}$	$(TACA)_{10}$
GenBank accession no.	CS693095	CS693096	CS693097	CS693098	CS693099	CS693100	CS693101[a]		CS693102	CS693103	CS693104	CS693105	CS693106

[a] MS6A and MS6B are included in one sequence since they are both found at a unique locus.

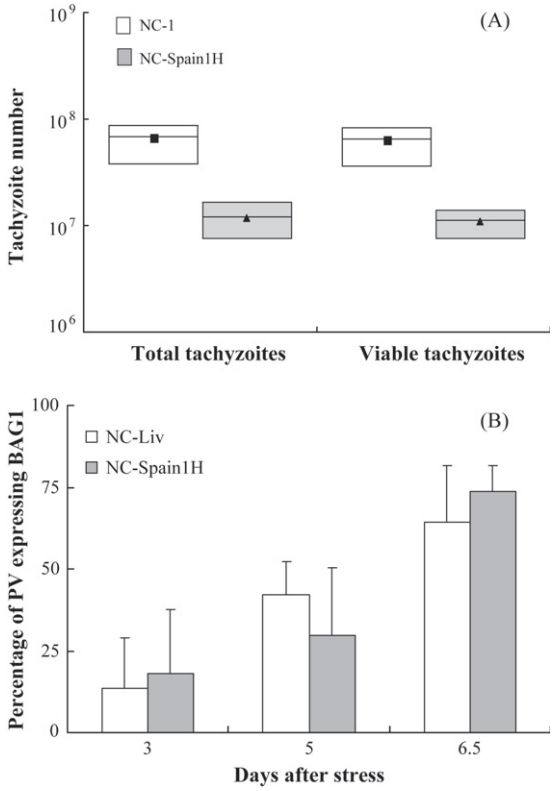

Fig. 1. Box-plot graph in (A) presents the lower and upper quartiles and the median yield of tachyzoites in cell cultures from two independent experiments performed in triplicate. In (B), the bars represent the mean percentage of PV expressing BAG1 for Nc-Spain 1H and Nc-Liv isolates from days 3 to 6.5 after stress, and error bars indicate the S.D. Values correspond to data from two independent experiments performed in triplicate.

1H and Nc-Liv isolates. Both isolates showed a high rate of bradyzoite conversion, with similar percentages ($P > 0.05$, Student's *t*-test) (Fig. 1B). However, 6.5 days after stress, the Nc-Spain 1H isolate produced only intermediate bradyzoites (SAG1- and BAG1-positive), whereas 3.4% of the PV in the Nc-Liv isolate contained pure bradyzoites (BAG1-positive). Once again, we observed that the monolayer infected with Nc-Liv was essentially destroyed 6.5 days after stress, but the monolayer infected with Nc-Spain 1H was hardly damaged.

3.3. Pathogenicity studies of the Nc-Spain 1H isolate in BALB/c mice

In experiment 1, 23.1% (6/26), 44.4% (12/27), and 37% (10/27) of the females inoculated with Nc-Spain 1H, Nc-1, and PBS, respectively, became pregnant. No differences in litter size were observed among the groups ($P > 0.05$, one-way ANOVA) (Table 2), but there was an increase in the number of stillborn animals in infected groups compared to uninfected mice, being significant only for the Nc-1 infected group ($P < 0.05$, χ^2). In addition, we found a higher number of PCR-positive stillborns in the Nc-1-infected group (72.7%, 8/11) compared to Nc-Spain 1H-infected (0%, 0/5) and uninfected mice (0%, 0/2). The

Table 2
Pregnancy rate, litter size, stillbirth, and vertical transmission in pregnant BALB/c mice inoculated with 2×10^6 Nc-Spain 1H or Nc-1 tachyzoites, or PBS buffer.

	Nc-Spain 1H	Nc-1	PBS
Pregnancy rate[a]	6/26 (23.1%)	12/27 (44.4%)	10/27 (37%)
Litter size[b]	4.2 ± 1.1	5.4 ± 1.7	5 ± 1.6
Stillborn[c]	5/25 (20%)	14/65 (21.5%)	2/45 (4.4%)
Vertical transmission[d]	1/20 (5%)	39/42 (92.8%)	0/43 (0%)

[a] Number of pregnant mice/total number of mated mice (percentage).
[b] Average ± S.D.
[c] Number of full-term dead pups at birth/total number of pups born (percentage).
[d] Number of positive PCR pups/total number of pups tested (percentage). Vertical transmission was calculated for the samples analyzed, as some samples could not be collected due to the cannibalism of dams.

offspring in the Nc-1-infected group showed significantly lower body weight compared to Nc-Spain 1H-infected and control mice from 14 to 20 days P.P. ($P < 0.05$, one-way ANOVA and Duncan's post-test) (Fig. 2). Pups born to Nc-1-infected mice showed clinical signs (delayed hair coat development, rough hair coat, and neurological signs) and a high neonatal mortality rate (76.8%, 43/56), in contrast to the Nc-Spain 1H-infected group, in which only one pup without clinical signs died during the observation period (0.5%, 1/20). No neonatal mortality was detected in control animals. The survival percentages of the Nc-Spain 1H infected group and PBS-inoculated mice were significantly greater than that of the Nc-1 infected group ($P < 0.001$, Log-rank test) (Fig. 3). Subsequently, Nc-1 infection produced the highest vertical transmission rate (92.8% of pups), and 100% of the dams transmitted the infection to at least one of the pups of their litter compared to the Nc-Spain 1H infected group, in which *N. caninum* DNA was detected in the brain of only one pup ($P < 0.0001$, χ^2) (Table 2). Among the dams, parasite DNA was detected in 75% (9/12) of the brain samples from Nc-1-infected mice and 33.3% (2/6) of those from Nc-Spain 1H-infected mice. With regard to the presence of parasites in the brains of mice that did not result in pregnancy, we detected *N. caninum* DNA in all samples from the Nc-1-infected group (100%, 15/15) and in only one sample from the Nc-Spain 1H-infected group (5%, 1/20). Regarding IgG2a and IgG1

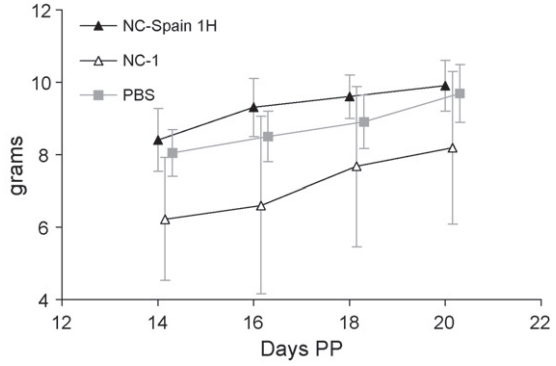

Fig. 2. Body weight of neonates born to dams inoculated with Nc-Spain 1H or Nc-1 tachyzoites, or PBS buffer. Each point represents the mean body weight of all animals in each group on days 14, 16, 18, and 20 P.P.

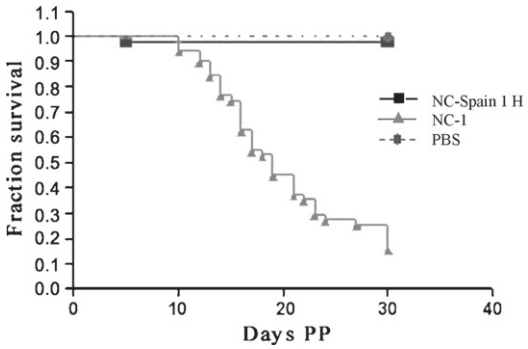

Fig. 3. Kaplan–Meier survival curves for neonates born to dams inoculated with Nc-Spain 1H or Nc-1 tachyzoites, or PBS buffer. The curves represent the percentage of animals surviving over a period of 30 days post-partum. Vertical steps downward correspond to days P.P. when a mouse was found death or was sacrificed. Symbols (■, ■, ▲) indicate censored observations. The number of dead mice was registered daily, and the percent survival of the Nc-Spain 1H-infected group and uninfected mice was significantly greater than that of the Nc-1-infected group ($P < 0.001$, Log-rank test).

responses, specific antibody responses were significantly higher in both infected groups than those in the non-infected group ($P < 0.001$, one-way ANOVA, Duncan's post-test). Among mice that became pregnant (Fig. 4A), the Nc-1-infected group showed significantly higher IgG1 and IgG2a levels than the Nc-Spain 1H-infected group ($P < 0.05$, one-way ANOVA, Duncan's post-test). Similarly, in mice that did not result in pregnancy (Fig. 4B), the Nc-1-infected group demonstrated higher IgG1 and IgG2a levels than Nc-Spain 1H-infected group, but only IgG2a levels

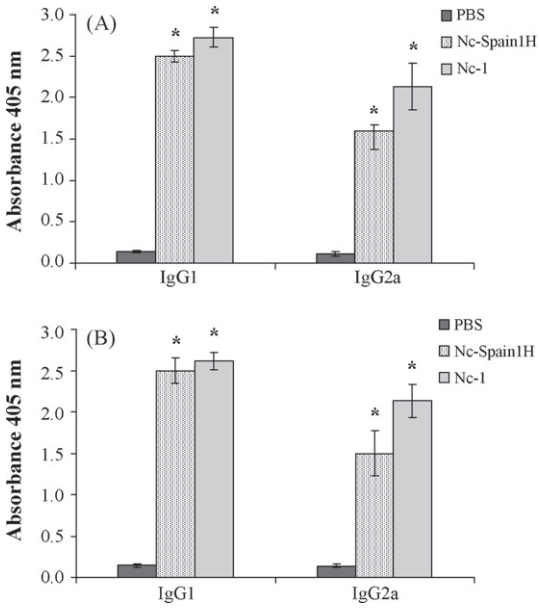

Fig. 4. Bars represent the mean absorbance, and error bars indicate the S.D. of anti-*N. caninum* IgG1 and IgG2a isotypes from pregnant BALB/c mice (A) and non-pregnant BALB/c mice (B) injected with Nc-Spain 1H, Nc-1 isolates, or PBS buffer. IgG1 and IgG2a antibody responses that were significantly different (*$P < 0.0001$, ANOVA test) compared to those for the non-infected group are denoted.

Table 3
Detection of *N. caninum* DNA by nested-PCR in blood, lung, and brain samples from BALB/c mice inoculated with 10^5, 10^6, or 10^7 Nc-Spain 1H tachyzoites.

Days p.i.	Blood[a]			Lung[a]			Brain[a]		
	10^5	10^6	10^7	10^5	10^6	10^7	10^5	10^6	10^7
1	1/3	3/3	4/4	0/3	0/3	3/4	1/3	1/3	1/4
2	1/3	1/3	4/4	0/3	3/3	4/4	1/3	1/3	3/4
4	1/3	1/3	4/4	0/3	3/3	4/4	1/3	2/3	3/4
8	0/3	0/3	0/3	1/3	1/3	3/3	0/3	0/3	1/3
16	0/3	0/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3
32	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

[a] Fractions represent the number of positive mice/total number of mice tested by nested-PCR.

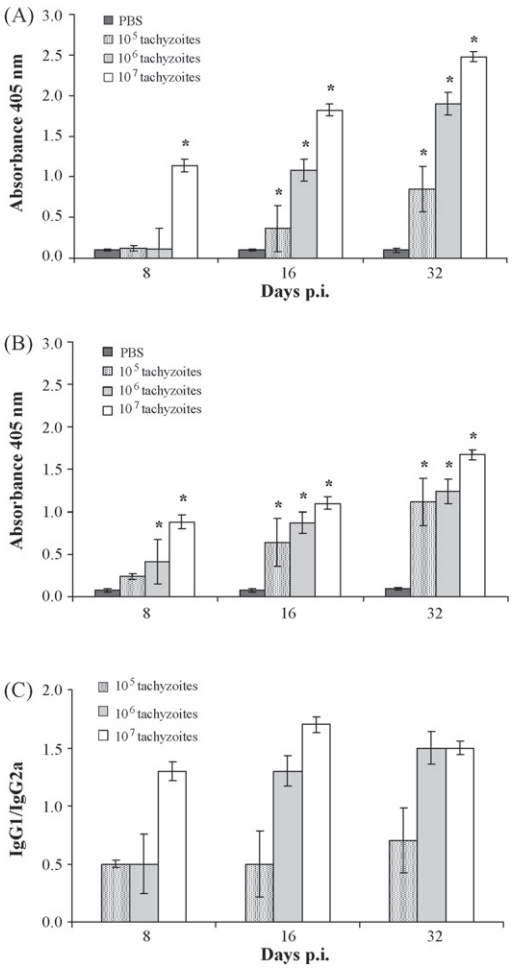

Fig. 5. Bars represent the mean absorbance, and error bars indicate the S.D. of anti-*N. caninum* IgG1 (A) and IgG2a (B) isotypes and ratio of IgG1/IgG2a (C) in BALB/c mice inoculated with 10^5, 10^6, or 10^7 Nc-Spain 1H tachyzoites or PBS buffer. In graphs (A) and (B), significant differences (*$P < 0.0001$, ANOVA test) in antibody levels compared with the non-infected group are denoted. Data corresponding to days 1, 2, and 4 p.i. are not represented because antibody responses developed by infected groups were not significantly different to those developed by control mice.

showed significant differences ($P < 0.05$, one-way ANOVA, Duncan's post-test). In both mouse models, all infected groups had a higher concentration of IgG1 than IgG2a (IgG1/IgG2a ratio >1), but no significant differences were observed between groups (data not shown).

In experiment 2, none of the infected mice showed clinical neosporosis or succumbed during the follow-up period. *N. caninum* DNA was observed in peripheral blood from 1 to 4 days p.i., in the lungs from 1 to 16 days p.i., and in the brain from 1 to 8 days p.i. (Table 3). The infection was dose-dependent, and higher doses resulted in increased parasite detection. Parasite DNA was not detected in target organs obtained from the control group. Regarding IgG2a and IgG1 responses (Fig. 5), mice inoculated with 10^7 tachyzoites demonstrated higher levels of IgG1 and IgG2a than the other groups on days 8, 16, and 32 p.i. ($P < 0.0001$, one-way ANOVA, Duncan's post-test). Mice inoculated with 10^5 tachyzoites had a higher concentration of IgG2a than IgG1 at all levels of infection (IgG1/IgG2a ratio <1), whereas, in the group inoculated with 10^6 tachyzoites, IgG2a predominated only at 8 days p.i. Groups inoculated with 10^7 tachyzoites showed a significant predominance in IgG1 levels (IgG1/IgG2a ratio >1) from 8 to 32 days p.i. (Fig. 5).

4. Discussion

Since the first isolation of *N. caninum* (Dubey et al., 1988), a limited number of isolates have been described and characterized. Moreover, most *N. caninum* studies have assessed isolates obtained from clinical cases. This could hamper our understanding of *N. caninum* pathogenesis, and isolate populations. *N. caninum* isolates of bovine origin have been obtained from aborted fetuses, stillborns, and apparently healthy animals (neonatal calves with precolostral *Neospora*-specific antibodies and two adult asymptomatic cows) (Dubey et al., 2007). It appears that the procedure utilized to isolate *N. caninum* from neural tissues of congenitally infected full-term calves is easier because tissue cysts, which are likely to be present, are relatively more resistant to autolysis than tachyzoites (Dubey and Schares, 2006). Based on this, we tried to obtain an isolate from a healthy congenitally infected calf with precolostral *Neospora*-specific antibodies. Obtaining and characterizing isolates from animals with different clinical forms of disease is necessary to evaluate correlations among parasite strains, genotypes, clinical manifestations, and pathogenicity. Furthermore, the identification of *N. caninum* avirulent isolates could lead to the development of live vaccines.

Identification and characterization of the new isolate was initially based on amplification and sequencing of the *N. caninum* ITS-1 region using a specific PCR test. ITS-1 sequence analysis has been used as an important tool for species characterization and differentiation between *Neospora* spp. and closely related parasites. The ITS-1 sequences obtained in this study identified the Nc-Spain 1H isolate as a member of the *N. caninum* species. Inter- and intra-strain variations in ITS-1 sequences between *N. caninum* isolates have been described (Gondim et al., 2004); however, they are not appropriate for intra-species differentiation. In contrast, microsatellite marker analysis proved to be a suitable tool for the genetic analysis and discrimination of *N. caninum* isolates (Regidor-Cerrillo et al., 2006). Thus, in the present study, 13 microsatellite markers were amplified and sequenced, and multilocus analysis showed that Nc-Spain 1H was genetically different from the other *N. caninum* isolates tested. In fact, two new alleles for microsatellites MS3 and MS6A were found. Analyses of the genetic diversity of *N. caninum* are limited. Further comparisons of isolates from different origins could be of considerable interest in determining how particular *N. caninum* genotypes might differ in their virulence and to facilitate predictions of infection outcome. In addition, the microsatellite technique could be useful in molecular epidemiological studies.

The antigenic specificity of the Nc-Spain 1H isolate was also confirmed by western blot using precolostral calf serum and plasma samples from mice inoculated with Nc-Spain 1H. When bovine sera and murine plasma samples were analyzed, immunodominant antigens of 17–18, 34–35, 37, and 60–62 kDa, which were previously identified (Álvarez-García et al., 2002), were recognized, and western blot revealed no major differences between the new Nc-Spain 1H isolate and the reference Nc-1 isolate. Similar profiles were described by Atkinson et al. (1999), who analyzed extracts from Nc-Liv and Nc-SweB1 isolates by western blotting with sera from experimentally infected mice.

With the aim of investigating the *in vitro* behavior of the Nc-Spain 1H isolate, we compared the tachyzoite yield of Nc-Spain 1H and Nc-1 isolates, since the *in vitro* growth rate and invasion efficiency are common virulence characteristics in protozoan pathogens, and growth rate differences between isolates have been previously described in both *N. caninum* (Schock et al., 2001; Pérez-Zaballos et al., 2005) and *T. gondii* (Saeij et al., 2005). A significantly higher tachyzoite yield was found for the Nc-1 isolate, and the number of plaque-forming tachyzoites counted in Nc-Spain 1H cultures was significantly lower compared to that of the Nc-1 isolate in the viability assay. Additionally, in both *in vitro* assays, we observed that Nc-1 destroys the monolayer faster than Nc-Spain 1H. Similar observations have been described for the three major strains of *T. gondii*, and this finding has been explained as due to a higher reinvasion rate of type I parasites. Extracellular type I parasites, regarded as "virulent", remain infectious for a longer period of time compared with type II or III strains (referred to as "avirulent"), and, consequently, they might be able to disseminate more efficiently to new cells after a previously infected cell is lysed (Saeij et al., 2005).

During *N. caninum* infection, parasite persistence is established in the CNS, where the parasite transforms into the tachyzoite–bradyzoite stage, which is walled off inside tissue cysts (Buxton et al., 2002). In order to determine the bradyzoite conversion rate of Nc-Spain 1H, an *in vitro* tachyzoite–bradyzoite stage conversion assay was performed. The stage conversion of Nc-Spain 1H observed in cell culture was similar to that seen in a cystogenic isolate of Nc-Liv, with the exception that Nc-Spain 1H produced only intermediate bradyzoites. This could potentially

hamper parasite persistence since the expression of specific pure bradyzoite antigens is a mechanism employed to evade the immune response induced against fast replicating tachyzoites during acute infection. Our *in vitro* observations correlate well with the results obtained in the *in vivo* assays. The low virulence of Nc-Spain 1H observed in mice could be explained by decreased parasite invasion of or a lower growth rate in host cells, which would lead to less efficient parasite dissemination.

Mouse inoculation was employed to determine the variation in virulence among *N. caninum* isolates. In previous studies, Nc-Liv was more virulent than Nc-1 and Nc-SweB1 (Atkinson et al., 1999; Quinn et al., 2002; Collantes-Fernández et al., 2006), and Nc-Nowra and JPA1 isolates obtained from healthy, but congenitally infected calves, were associated with low virulence in mice (Shibahara et al., 1999; Miller et al., 2002). We evaluated parasite virulence in a pregnant mouse model (López-Pérez et al., 2006, 2008). An increased number of stillborn pups were observed in infected mice, but parasite DNA was found only in stillborns from the Nc-1-infected group, suggesting that they may have died as a consequence of infection. We were unable to detect parasite DNA in the stillborn samples obtained from the Nc-Spain 1H group. Moreover, infection with Nc-1 during pregnancy caused high transplacental transmission, leading to a high neonatal mortality rate over time. In contrast, offspring from Nc-Spain 1H-infected dams remained clinically normal, the survival rate was almost 100%, and *N. caninum* DNA was detected in only one pup. In addition, parasite DNA was more often detected in brain samples from Nc-1-infected dams than Nc-Spain 1H-infected dams. Among those mice that did not become pregnant, parasite DNA was detected in all animals infected with the Nc-1 isolate and only one of those infected with the Nc-Spain 1H isolate. In another study, the Australian isolate Nc-Nowra was described as a low virulence isolate, although, when an inoculum of 10^6 tachyzoites per mouse was used, mortality was observed in 3 out of 10 infected mice and parasite DNA was detected in brain samples. Moreover, in a pregnant mouse model, the vertical transmission rate was high (87%) (Miller et al., 2002) with inocula lower than 2×10^6 tachyzoites per mouse, similar to the vertical transmission observed during infection with the Nc-Liv isolate using the same mouse model (91%) (Quinn et al., 2002). Significant *N. caninum*-specific antibody production was detected in the infected groups in both mouse models, with a dominant IgG1 response observed. The antibody levels varied with the isolate administered; inoculation of Nc-Spain 1H induced a lower antibody response than inoculation of Nc-1. The smaller antigenic stimulus produced by the Nc-Spain 1H isolate may be related to reduced parasite burden in mouse tissues. Variations in the immune responses of mice depending on the isolate inoculated have also been reported in a previous study, which suggested that this may be due to differences in the behavior of the isolates in a host (Quinn et al., 2002).

In the present study, we also used a BALB/c mouse model previously developed in our laboratory in which experimental infections with Nc-Liv and Nc-1 were characterized by an early phase of infection during which parasitemia and parasite DNA were observed in several murine organs, mainly the lungs, and by a chronic stage in which parasites were detected in the brain (Collantes-Fernández et al., 2006). Results obtained from experimental Nc-Spain 1H infections in mice demonstrated that this isolate failed to induce clinical signs or mortality and resulted in reduced burden, which was undetectable using our technique, or no parasite presence in the brain during the chronic stage, even in those mice inoculated with 10^7 tachyzoites. We also studied *N. caninum* antibody responses and showed that IgG2a and IgG1 isotype levels differed depending on the infectious dose administered. Mice inoculated with 10^5 tachyzoites showed a dominant IgG2a response, which might correlate with a cell-mediated response that appears to limit the multiplication of the parasite. In addition, an increased inoculum size led to increased IgG1 levels. For the intracellular protozoa *Leishmania*, parasite load is suggested to affect the type of immune response developed; a high parasite load would favor a Th2 response, which directly downregulates Th1 cells (Hondowicz and Scott, 2002).

Our results suggest that Nc-Spain 1H is a low virulence isolate with a low capacity for multiplication in host tissues and for transplacental transmission. However, the isolate was obtained from a congenitally infected calf, and it is likely that the parasite was transmitted from the mother to the fetus. Additional studies are necessary to investigate the pathogenicity of Nc-Spain 1H in bovines.

This paper describes the successful isolation and characterization of an *N. caninum* isolate from an asymptomatic naturally infected calf, and its low virulence in BALB/c mice. This work may lead to further studies regarding the biological diversity among isolates and their association with the presentation of disease in cattle. Furthermore, it could constitute a successful approach for the identification of low virulence *N. caninum* isolates for use as live vaccine candidates.

Acknowledgements

We thank Louis M. Weiss (Albert Einstein College of Medicine, New York) for the polyclonal rabbit anti-recombinant BAG1 (*T. gondii*) serum, Andrew Hemphill (Institute for Parasitology, University of Berne, Switzerland) for providing the monoclonal mouse anti-recombinant SAG1 (*N. caninum*), Diana Williams (Liverpool School of Tropical Medicine, Liverpool, U.K.), who kindly provided the Nc-Liv isolate, and Arvid Uggla (Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden) for the Nc-SweB1 isolate. We would also like to thank Vanesa Navarro Lozano for her excellent technical assistance. All mouse handling procedures complied with EU legislation. This work was supported by a project (PETRI 95-0777.OP) from the Spanish Government and Hipra Laboratories S.A. The Nc-Spain 1H isolate has been patented by the SALUVET group and HIPRA Laboratories S.A. (P200505316).

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Capítulo V

Vet. Res. (2009) 40:49
DOI: 10.1051/vetres/2009032

www.vetres.org

Original article

Experimental infection with a low virulence isolate of *Neospora caninum* at 70 days gestation in cattle did not result in foetopathy

Silvia Rojo-Montejo, Esther Collantes-Fernández, Javier Blanco-Murcia, Antonio Rodríguez-Bertos, Verónica Risco-Castillo, Luis Miguel Ortega-Mora*

SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain

(Received 27 February 2009; accepted 3 June 2009)

Abstract – The Nc-Spain 1H isolate of *Neospora caninum*, which was newly obtained from the brain of a congenitally asymptomatic infected calf, demonstrated a reduced in vitro tachyzoite yield and viability rate, as well as low virulence in mouse models. The objective of the present study was to determine the ability of this isolate to induce foetal death in a pregnant bovine model. For this purpose, 13 naïve pregnant heifers were divided into three groups and were experimentally challenged with either 10^7 tachyzoites of Nc-1 (group 1, $n = 5$), Nc-Spain 1H (group 2, $n = 5$) isolates or phosphate-buffered saline (group 3, $n = 3$) intravenously at 70 days of gestation. After inoculation, pregnancy was monitored and dams were sacrificed when foetal death was detected. The remaining animals were slaughtered at 45 days post-infection. Maternal and foetal samples were collected for examination by histology and parasite DNA detection. Parasitaemia, specific anti-*N. caninum* IgG and interferon γ responses were also studied. At 3–4 weeks after infection, foetal death was detected in 3 out of 5 Nc-1-infected dams. However, no evidence of foetal death was observed in either Nc-Spain 1H-infected or control groups during the period studied. The most severe histopathological lesions were observed in the placenta and foetal organs from Nc-1-infected cattle that exhibited foetal death. It was in these animals that *N. caninum* DNA was more frequently detected. Parasitaemia was observed in all Nc-1-infected dams and in only 3 out of 5 Nc-Spain 1H-infected animals. The magnitude of the immune response was significantly higher in the Nc-1-inoculated group than in the group inoculated with the Nc-Spain 1H isolate. These data reveal the reduced virulence of the Nc-Spain 1H isolate in cattle.

***Neospora caninum* / low virulence isolate / cattle / foetopathy / Nc-Spain 1H**

1. INTRODUCTION

Neospora caninum is a heteroxenous, cyst-forming coccidian closely related to *Toxoplasma gondii* that has been recognised as a major cause of abortion and reproductive failure in cattle worldwide [15]. Dogs and coyotes have been identified as definitive and intermediate hosts of the parasite, whereas cattle and other mammals are natural intermediate hosts [14]. The routes of *N. caninum* transmission in cattle include transplacental infection through tachyzoites, from the dam to the foetus during gestation (vertical transmission), and infection by ingestion of sporozoite-containing oocysts shed by a definitive host (horizontal transmission). Exogenous transplacental transmission occurs following primary oocyst-derived infection of dams,

* Corresponding author: luis.ortega@vet.ucm.es

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while endogenous transplacental transmission occurs following recrudescence of infection in persistently infected cows during pregnancy [33].

The consequences of either primoinfection or recrudescence in a pregnant cow can be abortion, birth of a weak calf or birth of a clinically healthy but persistently infected calf [9, 19]. A key question in understanding the variation in clinical presentation and severity of disease is the influence of the isolate. Biological diversity has been reported among some isolates of *N. caninum* in experimental murine infections [2, 10, 21] and in vitro studies [29, 31]. However, nothing is known about the differences in virulence of *N. caninum* isolates in cattle and how isolates with different virulence in mice may cause different disease outcomes in cattle. Experimental infection in cattle is necessary to confirm the correlations between parasite isolates with different virulence in in vitro and murine models, and clinical signs of disease in the natural host.

Recently, a new isolate of *N. caninum*, named Nc-Spain 1H, was obtained from the brain of a congenitally infected calf. This isolate was less virulent than the Nc-1 isolate in pregnant and non-pregnant mouse models and had a decreased rate of multiplication in in vitro cell culture [30]. Previous experimental studies in cattle have demonstrated that the inoculation of Nc-Liv or Nc-1 tachyzoites at day 70 of gestation induced foetal death [22, 34]. Using this protocol, we examined the ability of Nc-Spain 1H to induce foetal death in cattle. In parallel, different clinical, pathological and parasitological parameters, as well as the immune response induced as a consequence of the infection, were also evaluated and compared to those caused by the reference isolate Nc-1.

2. MATERIALS AND METHODS

2.1. Animals and experimental design

Thirty-one Holstein Friesian female cattle, aged 16–24 months and seronegative for *N. caninum*, *Leptospira* spp., Infectious Bovine Rhinotracheitis Virus and Bovine Viral Diarrhoea Virus were selected. These animals were oestrus synchronised using synthetic $PGF_{2\alpha}$ analogue (Prosolvin, Intervet, Salamanca, Spain) at 11 days intervals and were artificially inseminated after 3 days on 2 successive days with semen from a *N. caninum*-seronegative bull. Pregnancy was confirmed by ultrasound scanning on days 35 and 57 after insemination and 13 pregnant heifers were selected for the experiment. Animals were maintained in an open housing system with a straw-bedded yard at Complutense University of Madrid, Spain. Cattle were fed concentrates and alfalfa hay twice a day and were allowed free access to fresh water.

Animals were randomly selected and allocated into three experimental groups. Groups 1 and 2 were inoculated intravenously (i.v.) with 10^7 tachyzoites of the Nc-1 and Nc-Spain 1H isolates, respectively, at 70 days of gestation (d.g.). Group 3 received an inoculum of phosphate-buffered saline (PBS) i.v. and was used as a negative control and a sentinel for adventitious infections.

Cattle were observed daily for 45 days post-infection (p.i.). Rectal temperatures were recorded daily from 2 days prior to challenge to 14 days p.i., then twice weekly from 14 days p.i. onward. Animals with temperatures above 39.5 °C were considered to be febrile. Transrectal ultrasonography was used to determine foetal viability. All heifers were monitored once weekly throughout the experimental period. When ultrasound examinations confirmed foetal death, dams were sacrificed by an intravenous overdose of embutramide and mebezonio ioduro (T-61, Intervet). The remaining animals were slaughtered at the end of the experimental period. This experiment was conducted in accordance with guidelines established by the current laws of animal protection in Spain.

2.2. Sampling

Blood samples were collected before inoculation and twice weekly thereafter until 41 days p.i. by coccygeal venipuncture in EDTA, plain evacuated and heparinised tubes for DNA extraction, serology and IFNγ analysis, respectively. Heifers were necropsied immediately after euthanasia and tissues were recovered aseptically. Brain and placenta from dams and brain, heart and liver from foetuses were placed in 10% formol saline or stored at −80 °C until use for histopathology or PCR analysis, respectively.

2.3. Parasites

Tachyzoites from the Nc-1 isolate [13] were propagated under new culture conditions using

MARC-145 cells. This shift from Vero cells to a new cell line was expected to minimise the effect of prolonged maintenance in Vero cells on the biological characteristics of this isolate [5]. Prior to inoculation, Nc-1 and Nc-Spain 1H tachyzoites were propagated in MARC-145 cell monolayers using standard procedures previously described [29]. The experiment was carried out using similar parasite passage numbers in MARC-145 cells for Nc-1 (passage no. 12) and Nc-Spain 1H (passage no. 10). Parasite viability and number were determined by trypan blue exclusion, followed by counting three aliquots in a Neubauer chamber. The infection dose per animal was adjusted to 10^7 tachyzoites and diluted in 5 mL of PBS. Parasites were administered to heifers within 1 h of harvesting from tissue culture.

Nc-1 tachyzoites used for DNA extraction and as an antigen in specific IFNγ detection and ELISA techniques were washed three times in sterile (PBS, pH 7.4). Host cell debris was separated by passing the mixture through a 25-gauge needle, followed by passage through PD-10 columns (Amersham Biosciences, Uppsala, Sweden). Cell-free Nc-1 tachyzoites were pelleted by centrifugation (600× *g*, 10 min) and frozen at −80 °C until use. To obtain *N. caninum* soluble protein antigen, purified tachyzoites were suspended in 1 mL of 10 mM Tris-HCl containing 2 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO, USA) and disrupted by sonication (Sonifier 450, Branson Ultrasonic, Danbury, CT, USA) in an ice-bath. Cell debris and unlysed cells were removed by centrifugation (10 000× *g*, 20 min, 4 °C). The protein concentration of the supernatant was quantified using the Micro BCA protein assay (Pierce, Rockford, IL, USA), then aliquoted and frozen at −80 °C.

2.4. *N. caninum*-specific IgG response

Blood samples were allowed to clot before centrifugation at 1500× *g* for 10 min and the serum was removed, aliquoted and stored at −20 °C until required. Serum samples were assayed for specific IgG antibodies using an *N. caninum* soluble extract antigen-based ELISA as previously described [1]. Serum samples were diluted 1:100 for testing. The anti-bovine IgG1 and IgG2 monoclonal mAb (Laboratorie Service International, France) was diluted 1:4000. Serum samples were analysed in duplicate and the mean value of the optical density (OD) was converted into a relative index percent (RIPC) using the following formula: RIPC = (OD_{405} sample − OD_{405} negative control)/(OD_{405} positive control − OD_{405} negative control) × 100. A RIPC value ≥ 8.2 indicates a positive result.

2.5. *N. caninum*-specific IFNγ responses

Heparinised blood samples were maintained at room temperature and were cultured within 2 h of collection from cattle with 100 μL of PBS (unstimulated control), concanavalin A (10 μg/mL; Sigma) to ensure the ability of the cells to respond to stimulation and secrete IFN-γ, and with *N. caninum* Nc-1 isolate soluble antigen (1 μg/mL), as described previously [16]. In order to assess IFNγ production, duplicate plasma samples were tested using a commercial ELISA kit (Bovigam IFNγ kit, CSL, Australia) as recommended by the manufacturer and using positive and negative controls provided with the kit. The results are expressed as OD values.

2.6. DNA extraction and PCR

Different samples from the maternal brain, placenta and foetal tissues were randomly selected and pooled. Then, 5–8 g of the pool were homogenised in sterile PBS (dilution 1:2) in a stomacher ("Masticator" IUL, Barcelona, Spain) for 2 to 5 min or minced using a sterile scalpel for placental tissue. Samples were aliquoted in different tubes and frozen at −80 °C. DNA was extracted using 3–5 different aliquots of 50 μL homogenised tissues or 15 mg of placental tissue samples using Real Pure Genomic DNA Extraction Kit (Durviz, Valencia, Spain) following the manufacturer's instructions. *N. caninum* DNA was obtained from 10^7 tachyzoites. DNA was extracted from 500 μL of EDTA-blood using Real Pure DNA Extraction SSS Kit (Durviz) following the manufacturer's recommendations. The concentration of DNA was determined by spectrophotometry and adjusted to 50 ng/μL. A total of 5 μL was used for PCR amplification.

Nested PCR on the internal transcribed spacer region of *N. caninum* was carried out with four oligonucleotides as described by Buxton et al. [8]. A secondary amplification product was visualised by 1.8% agarose gel electrophoresis and ethidium bromide staining. DNA equivalent to 10^2 tachyzoites were used as the positive PCR control. To avoid false positive reactions, DNA extraction, PCR sample preparation and electrophoresis were performed in separate rooms employing different sets of instruments, aerosol barrier tips and disposable gloves. Moreover, negative control samples were included in each set of DNA extractions and PCR reactions.

2.7. Histopathological examination

A histopathological study was carried out on different sections from the brain and placenta from dams and brain, heart and liver from foetuses using routine histological methods. Tissues were fixed in 10% neutral buffered formalin and dehydrated through graded alcohols before being embedded in paraffin wax and stained with haematoxylin and eosin. The analysis was based on the observation of lesions according to previous descriptions [3, 28, 37], and lesions were classified as none detected/unrelated (−), consistent with (+), or characteristic of (++) bovine neosporosis.

2.8. Statistical analysis

Rectal temperatures, serology and IFNγ data were analysed using the one-way ANOVA test. When statistically significant differences were found, a Duncan Multiple Range test was applied to examine all possible pairwise comparisons. Lesion severity was analysed by the Mann-Whitney *U*-test. A *p*-value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Clinical observations

There was no significant increase in the mean temperatures from the different groups in the experiment. However, two animals from group 1 showed sporadic febrile responses at 2, 5, 13, 19 and 31 days p.i. The highest values were observed in one animal at 19 and 31 days p.i. (40.2 °C and 39.9 °C, respectively). In group 2, a febrile response was also observed in two heifers at 2, 5 and 26 days p.i., with 39.6 °C the highest temperature in this group.

Following infection at 70 d.g., foetal death was detected in 3 out of the 5 heifers in group 1 at 26 and 34 days p.i. The rest of the foetuses remained viable until the end of the experiment (Tab. I). Other clinical signs were not observed in any animal of the examined groups.

Table I. Detection of parasite DNA and histopathological changes in foetal and maternal tissues of cattle inoculated with Nc-1 (group 1) and Nc-Spain 1H (group 2) or PBS (group 3) at 70 days of gestation.

Group	Foetal death (days p.i.)	Foetal						Maternal			
		Liver		Heart		Brain		Placenta		Brain	
		HP[a]	DNA[b]	HP	DNA	HP	DNA	HP	DNA	HP	DNA
1	34*	++	3/3	++	3/3	+	3/3	++	3/3	+	–
	34*	+	1/3	++	3/3	+	3/3	++	3/3	–	–
	–	+	–	+	–	+	–	–	–	+	–
	–	+	–	+	–	+	–	+	–	–	–
	26*	++	3/3	++	3/3	+	3/3	++	3/3	+	–
2	–	–	–	+	–	+	–	–	–	–	–
	–	–	–	+	–	+	–	+	1/3	–	–
	–	–	–	+	–	+	–	–	–	–	–
	–	–	–	+	–	+	–	–	–	–	–
	–	–	–	+	–	+	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–	–
	–	–	–	–	–	–	–	–	–	–	–
	–	–	–	–	–	–	–	–	–	–	–

* Day p.i. when foetal death was detected by transrectal ultrasonography. The remaining foetuses lived until the end of the experiment.

[a] Histopathological lesion severity: none detected (−), consistent with (+), and characteristic of (++) *N. caninum* infection.

[b] Fractions represent number of samples positive by nested PCR/number of samples examined.

3.2. *N. caninum*-specific IgG response

The *N. caninum*-specific antibody response throughout the experiment is shown in Figure 1. In the Nc-1-infected group, the antibody concentration was significantly higher than in the uninfected group from 17 days p.i. onward ($p < 0.05$; one-way ANOVA, Duncan Multiple Range test) and it peaked at 24 and 26 days p.i. In the Nc-Spain 1H-infected group, the antibody level increased from 17 days p.i.; the highest value was detected at 19 days p.i. and decreased afterwards. The values did not vary significantly when compared to the uninfected group ($p > 0.05$; one-way ANOVA, Duncan Multiple Range test). When both infected groups were compared, group 1 had significantly higher antibody levels than group 2 on day 17 p.i. and from 24 days p.i. onward ($p < 0.05$; one-way ANOVA, Duncan Multiple Range test).

3.3. *N. caninum*-specific IFNγ response

Significant higher levels of IFNγ were detected in both infected groups from 5 to 12 days p.i. than in the uninfected group ($p < 0.05$; one-way ANOVA, Duncan Multiple Range test) (Fig. 2). In the Nc-1-infected group, the IFNγ levels remained significantly higher than in the uninfected group until the end of the study, with the highest value detected on day 24 p.i. ($p < 0.05$; one-way ANOVA, Duncan Multiple Range test). In the Nc-Spain 1H-infected group, the IFNγ values decreased to levels that were not significantly different compared to the uninfected group from day 12 p.i., excluding 19 and 34 days p.i. ($p < 0.05$; Duncan Multiple Range test). When we compared both infected groups, group 1 had significantly higher IFNγ levels than group 2 from 10 days p.i. onward ($p < 0.05$; Duncan Multiple Range test), except at 26 days p.i. when these differences were not significant.

3.4. Detection of *N. caninum* DNA in blood and tissues by PCR

Parasitaemia was detected sporadically in all heifers from group 1 on days 5, 12, 19 and 24 p.i. and in 3 out of 5 heifers from group 2 on days 3, 5, 19 and 41 p.i. (Tab. II).

N. caninum DNA was detected in the placenta, foetal brain, heart and liver from animals

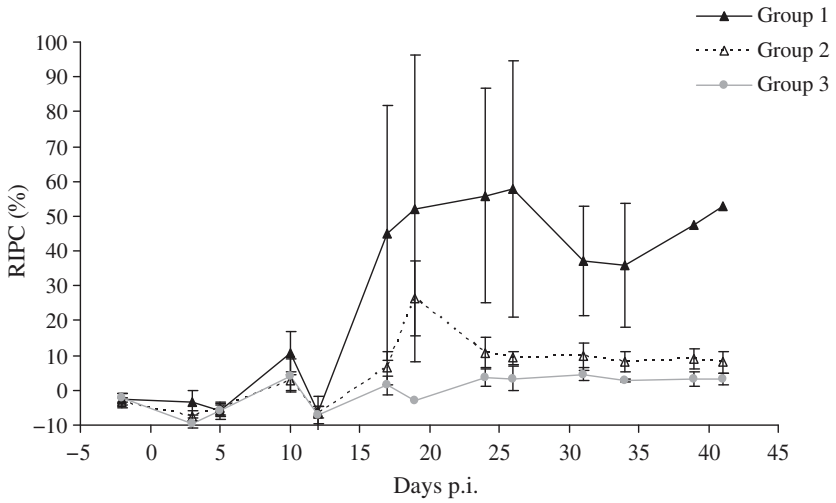

Figure 1. *N. caninum*-specific IgG responses in the serum of cattle inoculated at 70 days of gestation with Nc-1 (group 1), Nc-Spain 1H (group 2) or PBS (group 3). Data are expressed as mean relative index percent (RIPC) and error bars are the standard deviations for each group. Positive cut-off ≥ 8.2 RIPC.

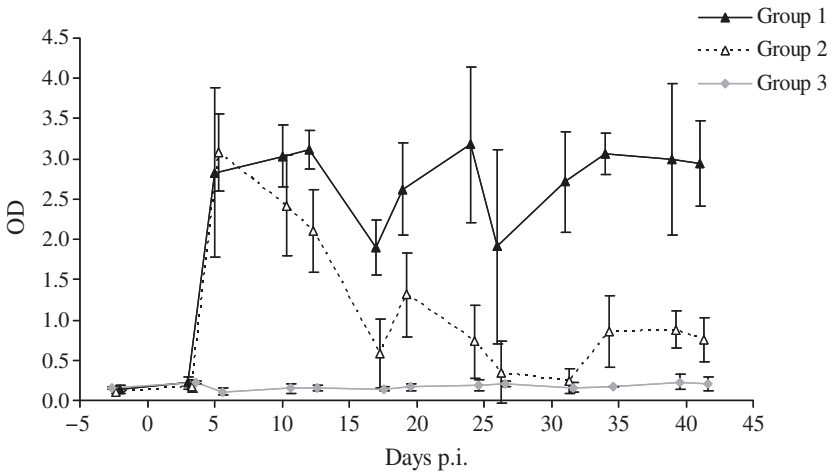

Figure 2. *N. caninum*-specific IFNγ response in cattle following inoculation at 70 days of gestation with Nc-1 (group 1), Nc-Spain 1H (group 2) or PBS (group 3). Data are expressed as mean optical density (OD) and error bars are the standard deviations for each group.

Table II. Detection of *N. caninum* DNA in the blood of animals inoculated at 70 days of gestation with Nc-1 (group 1) and Nc-Spain 1H (group 2).

Days p.i.	Group 1					Group 2				
	1 767	1 785	1 587	1 786	7 848	1 711	1 638	1 563	1 782	1 965
3	−	−	−	−	−	−	−	−	−	+
5	−	−	+	+	−	−	−	−	−	+
10	−	−	−	−	−	−	−	−	−	−
12	−	−	−	−	+	−	−	−	−	−
17	−	−	−	−	−	−	−	−	−	−
19	−	+	−	−	−	−	−	−	+	−
24	+	−	−	−	−	−	−	−	−	−
26	−	−	−	−	−	−	−	−	−	−
31	−	−	−	−	−	−	−	−	−	−
34	−	−	−	−	−	−	−	−	−	−
39	−	−	−	−	−	−	−	−	−	−
41	−	−	−	−	−	+	−	−	−	−

\+ *N. caninum* DNA was detected by nested PCR.
− *N. caninum* DNA was not detected by nested PCR.

that had presented foetal death in group 1. In group 2, parasite DNA was detected in the placenta from only one animal (Tab. I).

3.5. Histopathology

The most significant pathological changes were observed in the placentomes from cattle in the Nc-1-infected group that presented foetal death (Tab. I). The lesions consisted of multiple foci of non-suppurative inflammatory infiltrates in both maternal and foetal mesenchyme, areas of haemorrhage and necrosis in the caruncular septa, groups of foetal villi with necrosis and cell debris and serum leakage between the maternal and foetal tissues. In contrast, mild

serum leakage in the caruncular septa was observed in only one heifer from the Nc-Spain 1H-infected group that also had detectable *N. caninum* DNA. In the brain, 3 out of 5 heifers from the Nc-1-infected group presented scarce focal perivascular mononuclear inflammatory cell cuffs in the brain. No pathological changes were seen in the tissues of the other animals. When the lesion severity was compared, our findings demonstrated that more severe lesions were observed in the brain and placenta from group 1 than in those from group 2 ($p < 0.05$ Mann-Whitney U test).

In the dead foetuses from the Nc-1-infected group, multiple foci of hepatocellular necrosis in the liver and lymphocytic myocarditis in heart were observed (Tab. I). The remaining foetuses from both infected groups presented a mild diffuse inflammatory infiltrate of mononuclear cells in the heart. Focal lesions consisting of perivascular cuffing of mononuclear cells and glial nodules were observed in all foetal brain tissue from both infected groups. When lesion severity was compared, more severe lesions were observed in the foetal liver and heart tissues from group 1 than in those from group 2, but the differences were significant only for the liver ($p < 0.05$ Mann-Whitney U test).

4. DISCUSSION

This study highlights the relevance of the *N. caninum* isolate virulence on the outcome of infection in cattle. For the first time, inoculation of cattle with two different isolates revealed differences in clinical, immunological and pathological responses. We examined the pathogenic effects of infection with Nc-Spain 1H early in gestation, using a pregnant bovine model in which i.v. inoculation of 10^7 tachyzoites of Nc-Liverpool at 70 d.g. results in foetopathy between 3 and 5 weeks after infection, as previously described [17, 34–36]. Foetal mortality was also reported between 28 and 56 days p.i. in all the animals i.v. inoculated with 5×10^8 Nc-1 tachyzoites at 70 d.g. [22]. In the present study, we observed that Nc-Spain 1H did not have the ability to induce foetal death in pregnant cattle whereas Nc-1 caused death in 3 out of 5 foetuses between 26 and 34 days p.i.

We monitored the experiment for 45 days after infection. This time appears to be long enough to evaluate foetal mortality since foetal death occurred earlier than 6 weeks p.i. with Nc-Liverpool and Nc-1 isolates [22, 34, 35]. In fact, Macaldowie et al. [22] studied the pathogenesis of *Neospora* infection at 70 d.g. and found that at 42 and 56 days p.i. the uterus from i.v. Nc-1-inoculated dams was empty, caruncles were reduced in size and the uterine epithelium had been largely restored, suggesting that foetal death occurred earlier than 42 days p.i. In contrast to the previous results, we did not detect 100% foetal mortality in the Nc-1-infected group and a febrile response was not induced after i.v. inoculation of both *N. caninum* isolates [18, 22]. These differences could be due to the inoculum size used in our study, which was lower than in previous studies with the Nc-1 isolate (10^7 instead of 5×10^8 tachyzoites) [18, 22]. Moreover, different temperature responses have been associated with the dose of parasite inoculum [23, 24].

In a previous study, Nc-Spain 1H showed a low tachyzoite yield and viability rate in vitro. Additionally, it appeared to be a low virulence isolate in mice since it failed to induce clinical signs or mortality in a non-pregnant mouse model and led to a low transplacental transmission and neonatal mortality rate in a pregnant mouse model [30]. The low rate of multiplication of Nc-Spain 1H tachyzoites might imply a low level of parasitaemia and a low risk of placental infection. Here, parasitaemia was observed in all Nc-1- and in 3 of 5 Nc-Spain 1H-infected animals, indicating that the circulation of parasites occurred in both infected groups. The differences in the frequency of detection may be related to the isolate characteristics. However, parasitaemia was sporadically found in both groups, presumably because of small parasite numbers or short periods of detectable parasitaemia, as others have described before [22, 24, 32], making variations between groups difficult to assess.

We observed significant differences in the pathological effects of infection depending

on the isolate inoculated. The most severe lesions were observed in the placenta from Nc-1-infected animals which showed foetal death. Additionally, parasite DNA was detected in placental samples from these animals. The lesions were similar to those reported in previous experimental studies [22, 25] and may have been critical in the foetal death observed in our study. On the contrary, only one heifer from the Nc-Spain 1H-infected group presented mild lesions and a reduced *N. caninum* DNA presence, indicating that a low parasite number reached the placenta. In the foetuses, lesions were more severe in the Nc-1-infected group than in the Nc-Spain 1H-infected group, with a significant difference observed in the liver. Previous reports suggest hepatocellular necrosis would be observed when the infection occurs with a high dose of parasites, for instance in foetuses aborted during epidemic episodes of neosporosis [37]. The severe pathological changes observed in not only the liver, but also in the heart and brain from the Nc-1-infected group, simultaneously with the presence of the parasite DNA in tissues, suggest an uncontrolled spread and multiplication of the parasite that could directly lead to death. On the contrary, slight lesions and no parasite DNA were observed in the Nc-Spain 1H-infected group, which could indicate a controlled infection that did not progress to a fatal outcome.

The immunological response provides evidence of exposure to infection, since all infected animals developed *N. caninum*-specific responses after parasite inoculation. Antibody levels increased from 17 days p.i. in both infected groups, following a similar pattern to those observed in several experimental infections [4, 18, 34, 35] but the magnitude of the parasite-specific IgG response in the Nc-Spain 1H-infected group was less than the response in the Nc-1-infected group. Similarly, parasite infection induced high levels of IFNγ production in both infected groups, suggesting that the maternal immune cell-mediated response was actively responding to the parasite. However, *N. caninum*-specific IgG and IFNγ values in the Nc-Spain 1H-infected group declined to similar levels to those observed in the uninfected group, whereas levels in the Nc-1-infected group remained high until the end of the experiment. This may be associated with the reduction, or absence, of a repeated antigenic stimulus and suggests a transient infection similar to experimental infections in sheep with the avirulent S48 *T. gondii* tachyzoites, in which a decline in detectable antibody from six weeks after infection suggested a short-lived infection [6]. It is unknown if the immune response developed in Nc-Spain 1H-infected animals was able to reduce parasite burden, limiting the tissue damage or if the low capacity of the isolate to multiply in host tissues may be associated with the reduction or absence of repeated antigenic stimulus. Further studies in order to comprehensively assess the variations in the immune response in cattle depending on the inoculated isolate are required.

Several recent papers have suggested that the mode of transmission (horizontal or vertical) could influence the infection [11, 12, 26]. There is evidence that variation in infection transmission exists in the field and parasite virulence could help to explain some of the epidemiological and clinical patterns of bovine neosporosis. When the parasite is transmitted from the dam to the offspring, a low virulence could be selected in order to preserve the success of transmission. Therefore, a correlation between low virulence and vertical transmission could be established. When the parasite exploits two hosts in succession in a predator-prey system, virulence may be beneficial, since the goal of the parasite is to kill the host and to increase the chances of ingestion and infection of the second host.

This study reports the characterisation of a naturally attenuated bovine *N. caninum* isolate in an experimental bovine model. The present results, together with those previously obtained in a pregnant mouse model [30], in which Nc-Spain 1H induced a low vertical transmission rate (5%) in comparison with the high transplacental transmission induced by Nc-1 isolate (92.8%), demonstrate that Nc-Spain 1H appears to be a low virulence isolate. Therefore, Nc-Spain 1H may be a suitable candidate for live vaccine development, similar to the approach that has been successful in developing a live (S48) *T. gondii* vaccine used

to prevent toxoplasmosis in farm livestock [7]. Another naturally attenuated isolate of *N. caninum*, Nc-Nowra, was previously selected as a potential vaccination candidate and was tested in an experimental mouse model [27] and pregnant bovine model [36]. Nc-Nowra induced a reduction in transplacental transmission and protected against foetal death, respectively. Furthermore, the Nc-Spain 1H isolate may be a safe candidate since its inoculation in early gestation does not produce foetal death. This information appears to be essential in the development of live parasite vaccines since a major drawback of such vaccines is safety concerns [15, 20]. In this sense, the administration prior to pregnancy, as suggest Williams et al. [36] with the Nc-Nowra isolate, and by another via administration (subcutaneous or intramuscular) could be a safe option for the Nc-Spain 1H live vaccine. Nevertheless, further studies are necessary to evaluate the protection induced by this attenuated isolate against abortion and congenital transmission of *N. caninum* infection.

Acknowledgements. We would like to thank Vanesa Navarro and Alberto Benito for their excellent technical assistance and Ricardo García de la Mata for statistical advice. We are grateful to Gorka Aduriz, (Instituto Vasco de Investigación y Desarrollo, NEIKER) for his collaboration in this study. This work was supported by a PETRI 95-0777.OP project from the Ministerio de Educación y Ciencia and Laboratorios Hipra S.A. and was part of the E.U. research collaboration COST-854. S. Rojo-Montejo was financed by PETRI 95-0777.OP.

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Capítulo VI

EVALUATION OF THE PROTECTION CONFERRED BY A NATURALLY ATTENUATED *Neospora caninum* ISOLATE AGAINST CONGENITAL AND CEREBRAL NEOSPOROSIS IN MICE

Silvia Rojo-Montejo[1], Esther Collantes-Fernández[1], Inmaculada López-Pérez[1], Verónica Risco-Castillo[1], Antoni Prenafeta[2], Luis Miguel Ortega-Mora[1*].

Author Affiliations:

[1]SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040, Spain.
[2]HIPRA. Avda de La Selva s/n, AMER 17170, Spain.

***Corresponding author:**

SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n. 28040-Madrid, Spain. Phone: +34 913944069. Fax: +34 913944098. LMOM: luis.ortega@vet.ucm.es

Manuscript in preparation

ABSTRACT

The parasite *Neospora caninum* is an important abortifacient agent in cattle worldwide. At present, the development of an effective and safe vaccine against bovine neosporosis is of great relevance. Recently, a new isolate of *N. caninum* (Nc-Spain 1H), which was obtained from the brain of an asymptomatic congenitally infected calf, exhibited non-virulent behaviour in mouse and bovine infection models. The aim of this study was to determine the safety and efficacy of Nc-Spain 1H when used as a vaccinal isolate in well-established BALB/c models of congenital and cerebral neosporosis. Mice were subcutaneously immunised twice at 3-week intervals and were challenged with 2×10^6 tachyzoites of the virulent Nc-Liverpool isolate. After immunisation with live Nc-Spain 1H tachyzoites, no parasitic DNA was detected in the dams' brains before challenge, indicating this isolate to be a safe vaccine candidate. The efficacy of the live vaccine was evaluated in the first experiment after the immunisation of mice with 5×10^5 live Nc-Spain 1H tachyzoites. This immunisation protocol significantly reduced the neonatal mortality to 2.4%, reduced the vertical transmission from 92.8% to 2.8% and completely limited the cerebral infection. These results were associated with a Th1-type immune response. In the second experiment, the effect of various immunising doses was established using ten-fold dilutions of the tachyzoites (from 5×10^5 to 5×10^1). In all the cases, congenital protection rates above 60% were observed, and the mice that were immunised with the lowest dose (5×10^1) presented the highest protection rate (86%). Moreover, low immunising doses of Nc-Spain 1H induced an IgG2a response, and high parasitic doses induced an IgG1 response. These results confirm the safety and the efficient protection that was conferred by Nc-Spain 1H against congenital neosporosis, even when the mice were immunised with low parasitic doses.

Key words: *Neospora caninum* / naturally attenuated isolate / Nc-Spain 1H / live vaccine / mice

1. INTRODUCTION

The obligate-intracellular protozoan parasite *Neospora caninum* is a major cause of reproductive failure in cattle worldwide. Currently, no effective measures to prevent abortion or the vertical transmission of the parasite are available. Immunoprophylaxis has been postulated as the most cost-efficient alternative to control bovine neosporosis [1]. Live vaccines have demonstrated the most promising results in terms of protection because these formulations can more effectively stimulate both humoral and cell-mediated responses [2]. However, live vaccines may present safety problems. Several procedures have been developed to obtain low-virulence *N. caninum* strains, such as temperature-sensitive mutants, irradiated tachyzoites and attenuated tachyzoites, through prolonged passage in tissue culture [3-5]. Naturally attenuated isolates of *N. caninum* obtained from asymptomatic infected animals have emerged in the last few years as feasible live vaccine candidates [6-9].

Recently, a new naturally attenuated *N. caninum* isolate (Nc-Spain 1H) was obtained from the brain of a congenitally infected calf and was demonstrated to be an avirulent isolate. Nc-Spain 1H demonstrated a lower rate of multiplication in cell culture and a lower *in vitro* invasive ability than did the Nc-1 isolate [8, 10]. The pathogenicity of Nc-Spain 1H was examined in BALB/c mice; the results revealed that Nc-Spain 1H failed to induce clinical signs of infection or mortality, and no parasites were detected in these mice. In a pregnant mouse model, the offspring survival rate from Nc-Spain 1H-infected dams was almost 100%, and *N. caninum* was detected in only one pup [8]. Furthermore, the inoculation of Nc-Spain 1H tachyzoites in cattle at 70 days gestation did not induce foetal death [11]. These data indicate that Nc-Spain 1H may be a low-virulence isolate and may be a suitable candidate for live-vaccine development.

In contrast, studies regarding the influence of dose on the protective response, which allow the optimisation of the number of live parasites inoculated per animal, could provide results that prove valuable to cost-efficient industrial production. Additionally, some reports have suggested the importance of the antigenic dose in the modulation of the immune response and thus the development of vaccines [12, 13]. The aim of this study was to determine whether protective immunity could be induced by immunisation with the Nc-Spain 1H isolate to prevent transplacental transmission and cerebral neosporosis in a well-established BALB/c mouse model. Furthermore, we measured the effect of various immunising doses on this protection.

2. MATERIALS AND METHODS

2.1. Parasites and parasite antigens

Live *N. caninum* Nc-Spain 1H [8] tachyzoites were used for the immunisation, and tachyzoites from the Nc-Liverpool isolate [14] were used for the heterologous challenge. Nc-Liverpool tachyzoites were propagated under new culture conditions using MARC-145 cells. This shift from Vero cells to a new cell line was expected to homogenise the cell passage in Nc-Liverpool [15]. Prior to the experiment, the Nc-Liverpool and Nc-Spain 1H tachyzoites were maintained *in vitro* by continuous passage in MARC-145 cell monolayers, as previously described [15], to ensure healthy and actively replicating parasites. The experiment was performed using the following parasite passage numbers in the MARC-145 cells: Nc-Liverpool (passage no. 12) and Nc-Spain 1H (passage no. 10). The parasite viability and numbers were determined by trypan blue exclusion, followed by counting three aliquots in a Neubauer chamber. The infection dose per mouse was adjusted to the required doses for immunisation or challenge in a final volume of 200 µl per mouse. The parasites were administered to the mice within 1 h of harvesting from the tissue culture.

Nc-Liverpool tachyzoites that were used for antigens were washed three times in sterile PBS (pH 7.4). Host cell debris was separated by passing the mixture through a 25-gauge needle, followed by passage through PD-10 columns (Amersham Biosciences, Uppsala, Sweden). Cell-free Nc-Liverpool tachyzoites were pelleted by centrifugation (600×g, 10 min) and frozen at −80 °C until use. To obtain *N. caninum* soluble protein antigens, purified tachyzoites were suspended in 1 mL of 10 mM Tris-HCl containing 2 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO, USA) and were disrupted by sonication (Sonifier 450, Branson Ultrasonic, Danbury, CT, USA) in an ice bath. Cell debris and unlysed cells were removed by centrifugation (10,000×g, 20 min, 4°C). The protein concentration of the supernatant was quantified using the Micro BCA protein assay (Pierce, Rockford, IL, USA), and the supernatant was aliquoted and frozen at −80°C.

2.2. Mice and experimental design

Female BALB/c mice were purchased from a commercial supplier (Harlan Interfauna Ibérica, Barcelona, Spain). The mice were fed ad libitum in a controlled environment that included light and dark cycles (12-h light:12-h darkness). All the protocols that involved animals were approved by the Animal Research Committee of the Complutense University, Madrid, Spain, in compliance with the proceedings described in the Regulation of Internal Regime for Animal Research Committee (published at BOUC, no. 2, at 9 February 2006) and the EU legislation (Council Directive 86/609/EEC).

The vaccine efficacy against congenital and cerebral neosporosis was tested using both pregnant and non-pregnant BALB/c mouse models, as previously described [16-18]. Initially, to determine the protective capacity of Nc-Spain 1H, the BALB/c mice were immunised with 5×10^5 live Nc-Spain 1H tachyzoites (experiment no. 1). Subsequently, to

examine the optimal immunising doses, the mice were inoculated with ten-fold diluted Nc-Spain 1H tachyzoites (5×10^5 to 5×10^1) (experiment no. 2). In both experiments, the mice were subcutaneously (s.c.) immunised twice at three-week intervals with live Nc-Spain 1H tachyzoites. Groups of non-immunised/non-challenged and non-immunised/challenged mice were included in each experiment. Three weeks after the booster immunisation, the BALB/c mice were allowed to mate for 96 h following the synchronisation of oestrus using the Whitten effect [19]. Day 0 of the pregnancy was defined as the first day that the females were housed with males. The mice were s.c. challenged with 2×10^6 Nc-Liverpool tachyzoites at mid-gestation (between days 6 and 10 of gestation). The pregnant animals were housed individually and were allowed to carry their pregnancies to term. The pups were evaluated daily from birth to day 30 postpartum (PP) for congenital neosporosis [13, 20, 21]. The dams and non-pregnant mice were evaluated for cerebral neosporosis during chronic infection until days 30 PP and 30 post-challenge, respectively [13, 20, 21], when all the mice were sacrificed. Brains from the pups and adult mice were removed aseptically and frozen at –80ºC until needed for DNA extraction.

2.3. Parameters evaluated for safety and efficacy

The safety of the various formulations was determined by daily observation of the mice for adverse reactions and by palpation for the presence of nodules at the inoculation sites on day 5 after the booster vaccination. The presence of parasite DNA in the brain samples from immunised mice was determined by PCR on day 5 after the second immunisation and prior to challenge.

To assess the protective efficacy against congenital neosporosis, litter size, neonatal mortality and vertical transmission were determined. The litter size was defined as the number of pups delivered per dam. The neonates were examined daily for morbidity and mortality. Neonatal mortality was defined as the number of dead pups from birth to day 30 PP. Vertical transmission of *N. caninum* was identified by the presence of parasite DNA in the brains of pups. Protective efficacy against cerebral neosporosis was analysed in the dams and mice that did not become pregnant, by determining the presence of *N. caninum* DNA in the brain. To determine the optimal immunising doses, we calculated the protection rate against congenital neosporosis; this protection rate was defined as the proportion of neonates that remained healthy until the end of the experiment, with no parasites detected in their brain samples.

2.4. DNA extraction and nested-PCR

The Real Pure Extraction genomic-DNA kit (Durviz, Valencia, Spain) was used to extract DNA from 10-20 mg of each host tissue and 10^7 *N. caninum* tachyzoites, according to the manufacturer's instructions. The amounts of DNA were measured spectrophotometrically, and the samples were diluted to a final concentration of 50 ng/µl. For the detection of parasite DNA, a nested-PCR was performed against the internal transcribed spacer (ITS1) region of *N. caninum*, using four oligonucleotides as described by Buxton et al.[22].

2.5. Cytokine analysis

The cellular immune responses that were induced by immunisation with 5×10^5 live Nc-Spain 1H tachyzoites (experiment no. 1) were determined prior to challenge. On day 5 after the second immunisation, five random animals from each group were sacrificed, and their spleens were aseptically extracted and immediately processed for splenocyte culture, as previously described [20]. Briefly, the splenocytes were suspended in RPMI 1640 culture medium (Biowhittaker, Walkersville, Md.) and were plated in 96-well plates at a concentration of 4×10^5 cells/well. The splenocytes were stimulated in triplicate with concanavalin A (ConA) (5 µg/ml), *N. caninum* tachyzoite soluble extract (10 µg/ml) or only with media (control group). The cells were maintained at 37ºC with 5% CO_2 for 72 hours. Next, the culture supernatants were collected by centrifugation and stored at -80ºC until cytokine analysis. A commercial ELISA kit (BD Bioscience, San Jose, CA, USA) was used to quantify IFN-γ, IL-4 and IL-10 cytokines in the supernatants, according to the manufacturer's instructions. The results were expressed in pg/ml.

2.6. Humoral immune response

The humoral immune response induced by inoculation with ten-fold diluted Nc-Spain 1H tachyzoites (5×10^5 to 5×10^1) (experiment no. 2) was measured prior to challenge. On day 5 after the second immunisation, five random animals from each group were sacrificed, their blood samples were collected by cardiac puncture, and the recovered sera were aliquoted and cryopreserved at -80 ºC until serological analysis. Serum levels of the *N. caninum*-specific IgG1 and IgG2a isotypes were measured. Briefly, 96-well plates were coated with soluble *N. caninum* tachyzoite antigens (0.5 µg in 100 µl/well), and diluted murine serum samples (1:100) and anti-mouse IgG2a or IgG1 antibody (1:5000; Southern Biotechnology, USA) were used as described previously [16, 23]. The ELISA results were expressed as the average absorbance values at 405 nm. The threshold value arbitrarily discriminating between 'positive' and 'negative' (cut-off) was defined by adding 3 standard deviations to the mean A_{405} value of sera from non-immunised/non-infected mice. The serum isotype balance was evaluated using the IgG1/IgG2a ratio.

2.7. Data analysis

Differences in rates were evaluated using the Chi-squared test or Fisher F-test. Postnatal mortality was analysed using the Kaplan-Meier survival method and the log-rank statistical test [24, 25]. ELISA data were analysed using one-way ANOVA followed by Tukey's multiple comparison test. A value of $P<0.05$ was considered significant. The statistical analyses were performed using the Statgraphics Plus v. 5.1 (StatPoint, Inc., Herndon, VA, USA) and the GraphPad Prism 5 v. 5.01 (San Diego, CA, USA) software.

The statistical probit method was used [26] to titrate the protective effect of the immunising dose. This method transformed the sigmoid dose-response curve to a straight line, which was analysed using a specialised linear regression model (probit link func-

tion), based on the probability that an immunised animal experienced ‘‘immunisation failure’’. Immunisation failure was recorded when an immunised animal developed severe clinical disease, died, or was born congenitally infected following a lethal heterologous Nc-Liverpool challenge. The analysis was conducted using the statistical package SPSS Inc. (Chicago, IL, USA).

3. RESULTS

3.1. The Nc-Spain 1H isolate was a safe live-vaccine candidate.

Neither local or systemic reactions nor nodules at the injection site were observed after each immunisation prior to challenge. Furthermore, neither *N. caninum*-related clinical signs nor parasite DNA in the brains of the mice that were sacrificed before the challenge were detected in any immunised group. These findings indicated that the inoculation with live Nc-Spain 1H tachyzoites was safe.

3.2. Immunisation with 5×10^5 live Nc-Spain 1H tachyzoites limited vertical transmission and cerebral infection.

The first experiment measured the capacity of the attenuated Nc-Spain 1H isolate to prevent the transplacental transmission of parasites to the progeny and to prevent the establishment of chronic infections in the brains of adult mice. Upon challenge at mid-gestation, a significantly longer median survival time was observed in neonates from the mice that had been immunised with 5×10^5 live tachyzoites (29 days) *versus* the non-immunised/challenged mice (20 days) ($P<0.001$; Log-rank test). The offspring from the immunised group exhibited a significant reduction in their postnatal mortality rate (2.4%, 1/41) compared with the mortality rate that was observed in the non-immunised/challenged group (84%, 42/50) ($P<0.0001$, Fisher's exact test). The vertical transmission was reduced from 89.1% (41/46) in the non-immunised/challenged group to 2.8% (1/44) in the immunised group ($P<0.0001$, Fisher's exact test). No significant differences in litter size were observed between the groups (data not shown). When the vaccine efficacy against cerebral infection was evaluated in the dams and non-pregnant mice, all the immunised mice remained clinically healthy throughout the study, and no parasite DNA was detected in their brain samples ($P<0.0001$, Fisher's exact test).

3.3. Cytokine response after immunisation with live Nc-Spain 1H tachyzoites.

IFN-γ, IL-10 and IL-4 cytokine production was determined in *N. caninum*-specific stimulated splenocytes from the mice immunised with 5×10^5 live Nc-Spain 1H tachyzoites prior to challenge (Figure 1). Splenocytes from the vaccinated mice secreted high levels of IFN-γ upon stimulation with *N. caninum* soluble antigen on day 5 after the booster. Detectable levels of IL-10 and IL-4 cytokines were also observed in immunised mice.

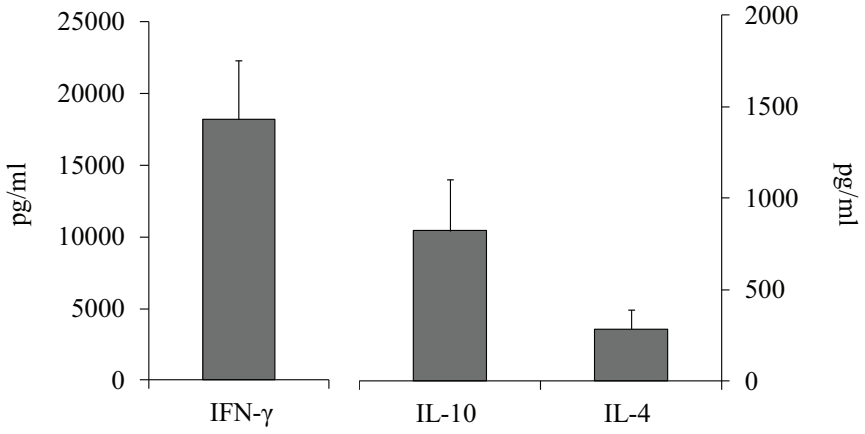

Figure 1. Cytokine production following stimulation with soluble *N. caninum* antigen in mice twice immunised with 5×10^5 live tachyzoites of Nc-Spain 1H on day 5 after booster. Bars represent the average amount of IFN-γ, IL-4 and IL-10 expressed in pg/ml, and error bars indicate the standard error of the mean (SEM) for each group.

3.4. Protection induced by live Nc-Spain 1H tachyzoites was not dose-dependent.

To investigate the influence of the parasite dose on protection, the BALB/c mice were twice immunised with 10-fold increased parasite doses (5×10^5 to 5×10^1) and then were challenged at mid-gestation (Table 1 and Figure 2). All the immunised groups exhibited more than 60% protection against congenital infection. These protection rates were significantly higher compared with the 17% protection in the non-immunised/challenged group (Table 1; $P<0.0001$, χ^2). Interestingly, the mice that were immunised with the lowest dose (5×10^1) showed the highest protection rate (86%). Concerning the dose-response relationship that was evaluated by the probit method, no significant differences between the probit values from the immunised groups were detected (Figure 3; $P=0.848$, probit). However, although no differences in the protection rates were detected, a weak trend toward a dose-dependent increase in protection was observed among the groups that were immunised with doses ranging from 5×10^2 to 5×10^5 live tachyzoites. Regarding the protection against cerebral infection, a significant reduction in the presence of parasite DNA in the brain samples was detected in all the immunised groups when compared to the non-immunised/challenged group (Table 1; $P<0.0001$, χ^2).

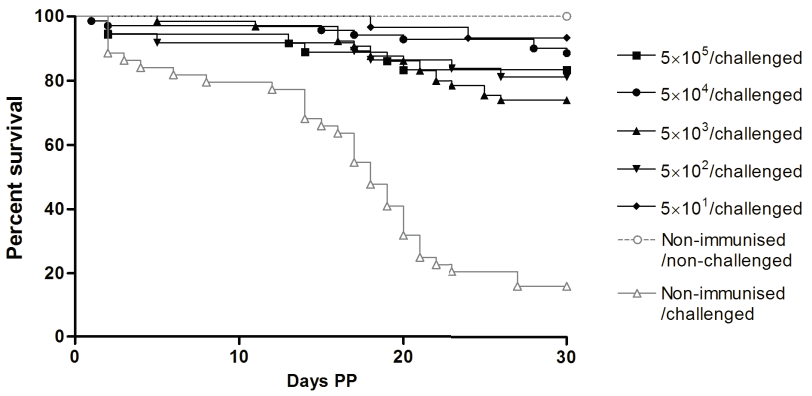

Figure 2. Kaplan–Meier survival curves for the neonates born from dams that were twice immunised with various doses of live Nc-Spain 1H tachyzoites (5×10^5-5×10^1). The curves present the percent survival as the proportion of all individuals over a period of 30 days PP. Vertical steps downward correspond to the days PP in which a death was observed. Symbols (■, ●, ▲, ▼, ◆, ○, △) indicate censored observations.

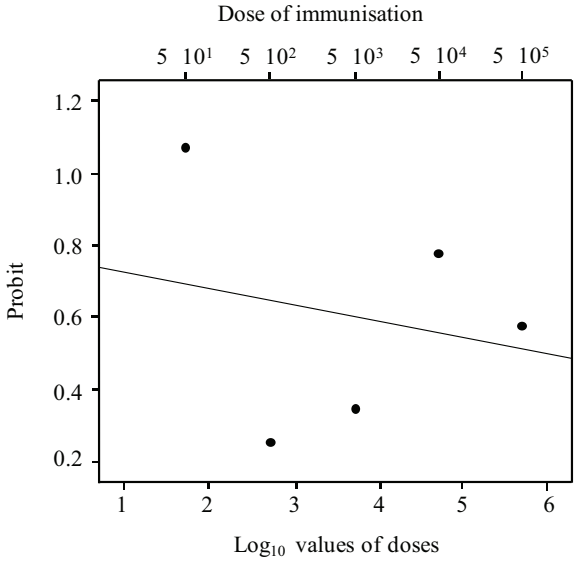

Figure 3. The probability of protection against congenital neosporosis, expressed in probit values that are plotted against the $\log_{10}$ immunising doses of Nc-Spain 1H tachyzoites in the dose-protection study. The fitting line represents a probit link function that estimates the expected probabilities of protection after immunisation with various doses of live Nc-Spain 1H tachyzoites ($\log_{10}$).

Table 1. Efficacy of various immunising doses of Nc-Spain 1H tachyzoites against congenital and cerebral infection in mice

Group	Congenital infection							Cerebral infection[h]
	Litter size[a]	Neonatal mortality		Vertical transmission		Protection rate[f]	Probit values[g]	
		Per pups[b]	Per litters[c]	Per pups[d]	Per litters[e]			
5×10^5/challenged	4 ± 1.9	6/36 (16.1%)	2/9 (22%)	6/39 (15.4%)	4/9 (44.4%)	28/39 (72%)	5.58	1/19 (5.26%)
5×10^4/challenged	4.7 ± 1.6	8/71 (11%)	6/15 (40%)	10/78 (12.8%)	6/15 (40%)	61/78 (78%)	5.77	0/24 (0%)
5×10^3/challenged	4.1 ± 1.9	17/65 (26%)	7/15 (47%)	29/90 (32.2%)	7/15 (47%)	59/93 (63%)	5.31	0/24 (0%)
5×10^2/challenged	3.7 ± 1.7	7/37 (19%)	6/10 (60%)	10/41 (24.4%)	5/10 (50%)	24/40 (60%)	5.25	0/26 (0%)
5×10^1/challenged	3 ± 1.7	2/30 (7%)	2/9 (22%)	2/41 (4.9%)	2/9 (22.2%)	36/42 (86%)	6.08	2/25 (8%)
Non-immunised/non-challenged	5.1 ± 1.6	0/61 (0%)	0/12 (0%)	0/64 (0%)	0/12 (0%)	0/64 (100%)	-	12/24 (50%)
Non-immunised/challenged	3.2 ± 2	38/45 (84%)	13/13 (100%)	48/65 (73.8%)	13/13 (100%)	11/65 (17%)	-	0/24 (0%)

[a] Average ± SD.

[b] No. of pups dead from birth to 30 days PP/no. of pups born alive (percentage).

[c] No. of litters with at least one pup dead from birth to 30 days PP/no. of litters in the group (percentage).

[d] No. of positive pups/no. of analysed pups (percentage).

[e] No. of litters with at least one positive pup/no. of analysed litters (percentage).

[f] No. of pups that remained healthy and with no parasites detected in their brain samples until the end of the experiment/no. of analysed pups (percentage).

[g] Probit values corresponding to the protection rates that were calculated from statistical tables of probit transformations.

[h] No. of nested PCR-positive adult mice/no. of analysed adult mice in the group at the chronic infection phase (percentage).

3.5. Antibody response following immunisation with live Nc-Spain 1H tachyzoites.

To determine the association between the pre-challenge antibody response and the immunising dose, we further determined which IgG antibody isotypes were increased. The mice that were twice immunised with 5×10^5, 5×10^4 and 5×10^3 tachyzoites exhibited the highest levels of IgG1 (Figure 4A; $P<0.0001$, one-way ANOVA; doses of 5×10^5, 5×10^4 and 5×10^3 tachyzoites vs. doses of 5×10^2 and 5×10^1 tachyzoites; dose of 5×10^5 tachyzoites vs. doses of 5×10^4 and 5×10^3 tachyzoites, Tukey's Multiple Comparison Test) and IgG2a (Figure 4A; $P<0.0001$, one-way ANOVA; doses of 5×10^5, 5×10^4 and 5×10^3 tachyzoites vs. dose of 5×10^1 tachyzoites, Tukey's Multiple Comparison Test). However, when the ratio IgG1/IgG2a was evaluated the groups that were inoculated with the lowest immunising doses induced a more polarised IgG2a response (IgG1/IgG2a ratios <1) (Figure 4B; $P<0.0001$, one-way ANOVA; doses of 5×10^5 and 5×10^4 tachyzoites vs. doses of 5×10^2 and 5×10^1 tachyzoites, Tukey's Multiple Comparison Test). These results indicate that low immunising doses of Nc-Spain1H promoted an IgG2a-biased response, whereas high parasite doses induced an IgG1-biased response.

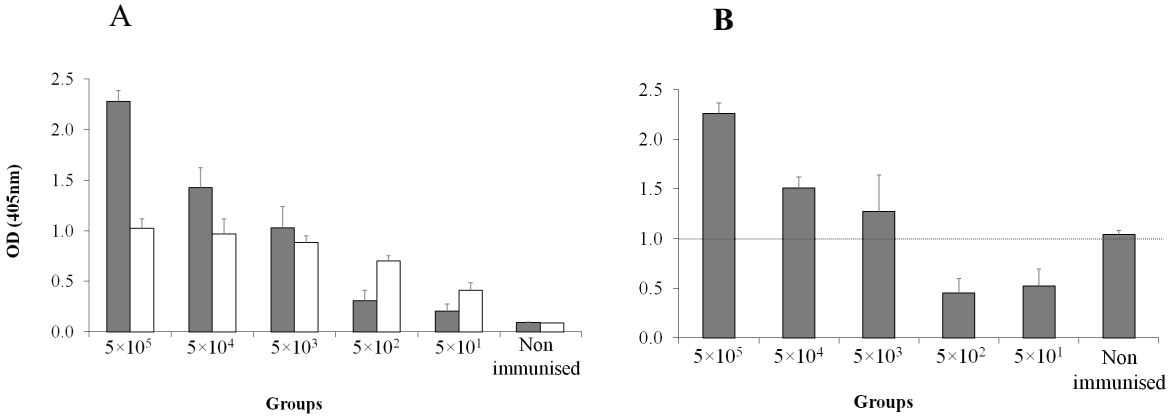

Figure 4. Panel A. ELISA using anti-*N.caninum* IgG1 and IgG2a from the BALB/c mice that were immunised with various doses (5×10^5-5×10^1) of Nc-Spain 1H at day 5 after booster (panel A). The bars represent the optical density (OD) at 405 nm, and the error bars indicate the SEM for each group. Positive cut-offs were established in ELISA for IgG1 detection at ≥ 0.132 and in ELISA for IgG2a detection at ≥ 0.131. A total of 5 mice prior to challenge were included in the analysis. **Panel B.** Bars represent the average of anti-*N. caninum* IgG1:IgG2a isotype ratios from the BALB/c mice that were immunised with various doses (5×10^5-5×10^1) of Nc-Spain 1H at day 5 after booster. Error bars represent the ± SEM. The discontinuous line marks identical IgG1 and IgG2a levels (IgG1:IgG2a = 1).

4. DISCUSSION

Vaccines provide green solutions to control disease because they are sustainable and reduce the reliance on pharmacological drugs and pesticides [2]. Live vaccines have been highly successful against protozoan parasites such as *Toxoplasma gondii*, in which vaccination with the live attenuated S48 strain prevents abortions in ewes [27]. In fact, Toxovax® is currently the only commercial vaccine for toxoplasmosis worldwide. In bovine neosporosis, one of the approaches is to identify isolates that are attenuated and can be used for live vaccine development. The naturally attenuated Nc-Nowra strain of *N. caninum*, which was isolated from an infected calf in Australia, has been previously tested as a potential live-vaccine candidate in an experimental mouse model [28] and a pregnant-bovine model [29], inducing a reduction in transplacental transmission and protection against foetal death, respectively. In spite of these promising protective results, it is currently unknown whether this isolate of *N. caninum* is responsible for foetal loss or is vertically transmitted to progeny in cattle [30]. This fact might be a major safety concern that would undermine the live-vaccine approach. Furthermore, mice that were immunised with different attenuated parasites similarly showed a reduction or completely prevention of brain pathology after challenging with virulent *N. caninum* parasites [3-5, 31].

The present study determined the vaccine efficacy and safety of a naturally attenuated *N. caninum* isolate using well-established mouse models of cerebral and congenital neosporosis [16-18]. The pregnant mice model provides a highly stringent tool for testing the efficacy of vaccines against the transmission of the parasite to progeny because there is a high transmission rate of *N. caninum* to the offspring after the inoculation of dams with a virulent isolate at the second trimester of gestation. The immunisation with 5×10^5 live Nc-Spain 1H tachyzoites conferred excellent protection against heterologous challenge with Nc-Liverpool tachyzoites, demonstrating reduced neonatal mortality (from 84% to 2.4%), reduced vertical transmission (from 92.7% to 2.8%) and complete protection against cerebral infection in adult mice. Moreover, the immunisation elicited a protective immune response characterised by a strong IFN-γ induction. It is widely reported that IFN-γ is one of the most critical cytokines, mediating host protection against the *N. caninum* infection by limiting parasite growth [32-34]. However, a suitable cytokine production of cellular and humoral immune responses may have an important role for the control of the *N. caninum* infection. Thus, the increased IL-4 and IL-10 levels might have been produced to restrain the inflammatory response and restore a balance in the immune response [35, 36]. Regarding the safety of the live vaccine, no parasite DNA was detected in the brains of the immunised mice. Additionally, in previous studies, Nc-Spain 1H was avirulent in pregnant and non-pregnant mouse models. In cattle, the inoculation of this isolate in early gestation does not produce foetal death [11]. Taken together, these results demonstrate that inoculation with live attenuated parasites is safe and generates a protective immune response against *Neospora,* suggesting that the Nc-Spain 1H isolate is a suitable candidate for a live vaccine.

Having established that immunisation with 5×10^5 live Nc-Spain 1H tachyzoites induced protection, we next addressed the effects of various doses, which would be valu-

able for optimising the immunising dose. The major obstacles during the commercial manufacture of a live vaccine are the short shelf life of viable parasites and the safety of the vaccine itself. Here, no differences were observed among the groups that were immunised with different doses. Protection against congenital neosporosis was high in all groups, and, unexpectedly, the lowest dose induced the highest protection level. The present study provides encouraging results regarding the possibility of reducing the administered dose of live Nc-Spain 1H tachyzoites. However, care must be taken when extrapolating from the mouse data, and the vaccine efficacy must be confirmed in the target species.

We also observed that the parasite dose appears to modulate immune responses. The mice that were administered the higher immunising doses predominantly produced IgG1, in contrast, low immunising doses of Nc-Spain1H promoted an IgG2a response. In other intracellular protozoan parasites, such as *Leishmania*, studies have provided evidence of the dose-dependent character of the acquired resistance that affects the Th1/Th2 nature of the immune response [37-39]. Some reports also suggest that low doses of parasites promote a Th1 response, whereas high parasite doses induce a Th2 response; in contrast, other studies suggest the opposite [38-40]. In a previous *N. caninum* study, Lundén et al. [41] demonstrated that the immunisation with a relatively virulent isolate at different subclinical doses of infection (10^4 and 10^6 tachyzoites) induced protective immunity in BALB/c mice, even though different antibody profiles were detected. Specifically, in mice given the highest number of parasites, the levels of IgG1 and IgG2a were equally high, while mice inoculated with the lower dose had higher IgG2a than IgG1 titres. Due to the intracellular nature of the *N. caninum* infection, the predicted protective immune response would be predominantly a type-1 (Th1) cell-mediated immune response dominated by the production of IFN-γ, IL-12 and IgG2a antibodies [32, 42]. However, it has also been suggested that a balanced Th1–Th2 response is required to limit a damaging host immune response. The inoculation of a low number of parasites may result in the internalisation of most of the tachyzoites inside the early antigen-presenting cells, enhancing an effective cell-mediated immunity. On the contrary, after the inoculation of a high parasite dose, some tachyzoites may remain extracellular, eliciting an IgG1-biased humoral response, that is adequate to control the extracellular parasites. Moreover, antibodies would block the invasion of the parasites into host cells, similar to what has been observed in other protozoan parasites [43, 44]. Nonetheless, from the results on antibody and cytokines production observed after immunisation with 5×10^5 live tachyzoites it cannot be concluded an association between protection and a clear-cut Th1 or Th2 response. Further studies are needed to investigate the mechanisms by which the parasite dose modulates the protective immune response.

In this paper, we report that the immunisation of mice with naturally attenuated tachyzoites of the Nc-Spain 1H isolate induced a protective immunity, which was able to efficiently control both congenital and cerebral neosporosis. All the immunising doses conferred protection against the vertical transmission of *N. caninum*, and this protective efficacy was not dose-dependent. These findings present the opportunity to test Nc-Spain 1H as a live vaccine candidate in cattle, using formulations containing low parasite doses;

this approach could have practical applications because it may favour the commercial manufacture of a live vaccine. Additionally, a critical component in designing effective vaccines is an understanding of the mechanisms by which the immune system is able to protect the host. Further studies should be conducted to address the duration of protection and the immunological mechanisms that are involved in protective immunity.

ACKNOWLEDGEMENTS

We thank Diana Williams (Liverpool School of Tropical Medicine, Liverpool, U.K.) for kindly providing the Nc-Liverpool isolate. We also thank Vanesa Navarro Lozano for her excellent technical assistance. This work was supported by projects from the Spanish Government and HIPRA (REF. PTR1995-0777.OP) and from the Complutense University of Madrid and HIPRA (Article 83, regulated by *Ley Orgánica 6/2001*, adopted 21 December 2001). Silvia Rojo Montejo was supported by a fellowship from Consejería de Educación (Comunidad de Madrid). The Nc-Spain 1H isolate has been patented by the SALUVET group and HIPRA (P2005053163; PCT/EP2006012296).

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Capítulo VII

Desde que en 1989 se describió por primera vez a *N. caninum* como agente causal del aborto bovino (Thilsted & Dubey, 1989), este patógeno se ha erigido como una de las principales causas de fallo reproductivo en esta especie doméstica, originando importantes pérdidas económicas en este sector pecuario a nivel mundial. Esto ha motivado que en los últimos 20 años se hayan llevado a cabo numerosas investigaciones dedicadas al desarrollo y mejora de estrategias de control de la neosporosis bovina. La única herramienta disponible en la actualidad es la aplicación de medidas de manejo del rebaño, que en muchas situaciones resultan inaplicables o insuficientes y, en todo caso, económicamente inviables. Es, por tanto, necesario implementar nuevas estrategias de control de la enfermedad que respondan a las necesidades del sector. En este sentido, la vacunación se ha sugerido como la alternativa más rentable, en comparación con otras medidas, aún cuando los niveles de protección frente al aborto no alcanzaran el 50% (Reichel & Ellis, 2006). Por ello, el desarrollo y comercialización de una vacuna eficaz y segura frente a la infección por *N. caninum* supondría un importante logro para el campo de la Sanidad Animal, en general, y para el sector bovino, en particular.

La mayoría de las investigaciones en el campo de la inmunoprofilaxis frente a la infección por *N. caninum* se han centrado en la obtención de vacunas de nueva generación basadas, principalmente, en proteínas recombinantes, vacunas de ADN y en la utilización de vectores vivos de expresión (Reichel & Ellis, 2009). Estas aproximaciones han mostrado ser seguras y permitirían inducir una respuesta inmunitaria dirigida a antígenos concretos que participan en procesos claves de la biología del parásito, pudiendo interferir en ésta. Sin embargo, el factor limitante en el desarrollo de este tipo de vacunas es la dificultad para identificar y seleccionar antígenos relevantes que estimulen una respuesta inmunitaria protectora frente a un parásito como *N. caninum* que exhibe un ciclo tan complejo. De hecho, los diferentes trabajos publicados hasta la fecha indican resultados de inmunoprotección insuficientes (Innes & Vermeulen, 2006; Reichel & Ellis, 2009). Por el contrario, las únicas vacunas que hasta el momento se han probado en la especie de destino con cierto éxito se basan en la utilización de parásitos inactivados o vivos atenuados obtenidos mediante técnicas clásicas.

Las vacunas inactivadas han sido ampliamente ensayadas con resultados poco prometedores. Estas vacunas se han elaborado, mayoritariamente, a partir de extracto soluble del parásito obtenido tras el lisado de éste (Innes & Vermeulen, 2006; Innes et al., 2011), pudiendo no incluir determinados antígenos inmunoprotectores que se encuentren en otras fracciones celulares. Los mejores resultados de protección frente a la neosporosis se han observado con el empleo de vacunas vivas atenuadas (Innes et al., 2011), probablemente asociado al hecho de que la inmunización con parásitos vivos es capaz de inducir una respuesta similar a la que tendría lugar tras una infección natural. Con estos antecedentes, el objetivo de la presente tesis doctoral consistió en el desarrollo de vacunas frente a la infección por *N. caninum,* en primer lugar, por medio de la utilización de parásitos enteros inactivados, examinando la influencia de diversas variables de interés como el adyuvante, tipo de antígeno o dosis antigénica -Capítulos II y III- y, en segundo lugar, mediante la obtención, caracterización y posterior empleo como vacuna viva de un aislado atenuado naturalmente - Capítulos IV, V y VI- .

Como primer objetivo de la presente tesis doctoral, se planteó el desarrollo de una vacuna inactivada frente a la neosporosis bovina. Debido a que este tipo de vacunas generan una respuesta inmunitaria menos potente que las vacunas vivas, los puntos clave son la selección de los antígenos del parásito y de los sistemas de liberación de éstos que favorezcan su correcto procesamiento y presentación al sistema inmunitario, de manera que induzcan una respuesta inmunitaria innata y adquirida protectora frente a la infección por *N. caninum* (Innes & Vermeulen, 2006). Por ello, durante el desarrollo del Subobjetivo 1.1 (Capítulo II) se procedió a la selección de diversas variables de interés, como el adyuvante y la dosis antigénica, en un modelo murino de neosporosis cerebral previamente estandarizado en nuestro laboratorio (Collantes-Fernández et al., 2006b). En dicho modelo, la infección subletal con 10^6 taquizoítos del aislado de referencia Nc-1 estuvo caracterizada por una fase aguda, de diseminación orgánica, y una fase crónica de transformación y acantonamiento del parásito en el cerebro. Siguiendo una aproximación de ensayo-error, este modelo experimental permitió evaluar la influencia del adyuvante o la dosis antigénica sobre la eficacia protectora durante la primera fase de la infección frente a la parasitemia y la distribución y multiplicación del parásito en diferentes órganos como el pulmón y, posteriormente, frente a la posible cronificación y persistencia de la infección en el cerebro.

Una variable que puede estar influenciando la eficacia de las formulaciones inactivadas es el tipo de antígeno utilizado. En este caso, se decidió inmunizar a los ratones con taquizoítos enteros inactivados. El uso de parásitos enteros que contienen antígenos de organelas, citoplasma y membrana celular, podría aportar beneficios sobre la utilización de extracto soluble del parásito utilizado en la mayor parte de preparaciones inactivadas que se han probado hasta la fecha. En este sentido, un estudio reciente ha señalado que la estimulación *in vitro* con parásito entero induce una respuesta inmunitaria desviada hacia el tipo Th1 mayor que la observada con parásito lisado (Feng et al., 2010). La inactivación de los taquizoítos se realizó con BEI, método químico que ya había sido utilizado por otros autores en la preparación de la única vacuna que hasta la fecha se ha registrado frente a la neosporosis bovina (Andrianarivo et al., 2000).

En el presente estudio se probaron tres adyuvantes (emulsión oleosa de agua en aceite -W/O- e hidróxido de aluminio combinado con CpG -Al/CpG- o extracto de Ginseng -Al/G-) en combinación con tres dosis de taquizoítos enteros inactivados (10^5, 5×10^5 y 10^6). Tanto el W/O como el hidróxido de aluminio se evaluaron en este estudio por su disponibilidad para ser administrados en vacunas de uso veterinario ya que han sido utilizados en numerosas formulaciones comercializadas frente a diversas enfermedades infecciosas que afectan a las especies de renta (Wilson-Welder et al., 2009). Su principal mecanismo de acción se basa en generar un reservorio del antígeno con el que se coadministra, favoreciendo su lenta liberación al organismo y una estimulación prolongada del sistema inmunitario. En numerosas ocasiones se ha descrito que dicho mecanismo favorece predominantemente la activación de una respuesta de tipo humoral (Aucouturier et al., 2001; Lindblad, 2004). Sin embargo, debido a la naturaleza intracelular de *N. caninum*, parece necesario emplear adyuvantes que favorezcan la estimulación de una respuesta inmunitaria mediada por células. Por ello, el hidróxido de aluminio se combinó con CpG

y con extracto de Ginseng. En el primer caso, el hidróxido de aluminio parece tener un efecto sinérgico que potencia las propiedades de los CpG (Gupta et al., 1995), desviando la respuesta inmunitaria hacia un tipo Th1 (Vasilakos et al., 2000). De manera similar, el hidróxido de aluminio combinado con extracto de Ginseng actúa sinérgicamente (Rivera et al., 2003), pudiendo favorecer el incremento de la actividad de las células del sistema polimorfonuclear fagocitario, la proliferación de linfocitos y la estimulación de citoquinas del tipo Th1 (IFN-γ o TNF-α) que estas saponinas inducen cuando se administran solas (Song & Hu, 2009).

El adyuvante W/O fue capaz de limitar la multiplicación del parásito en el cerebro durante la fase crónica de la infección. Esta protección estuvo asociada con una elevada producción de anticuerpos tanto Th1 como Th2, sugiriendo el desarrollo de un equilibrio de la respuesta inmunitaria que pudo favorecer el control de la infección. Por el contrario, el adyuvante Al/CpG indujo sólo una ligera reducción en la presencia y carga del parásito en el cerebro de los animales vacunados. Numerosos estudios han descrito el efecto protector del adyuvante CpG en organismos intracelulares, incluido *N. caninum* (Jenkins et al., 2004; Ribeiro et al., 2009), asociado a la activación de una respuesta inmunitaria de tipo Th1. La combinación Al/CpG, sin embargo, indujo unos bajos niveles de anticuerpos con un predominio del isotipo IgG1. Aunque se desconoce el mecanismo de acción del Al/CpG, la escasa protección conferida por esta combinación podría estar asociada a una respuesta de tipo Th2, asociada principalmente a la administración de las sales de aluminio empleadas en este estudio. Por último, el adyuvante Al/G limitó la parasitemia durante la fase aguda. Sin embargo, durante la fase crónica no protegió frente a la infección cerebral, observándose una exacerbación de la infección tras la inmunización con Al/G en combinación con el antígeno. La magnitud de la parasitemia podría estar relacionada con el grado de diseminación orgánica del parásito, reduciendo su llegada y multiplicación en los diferentes tejidos, y las consecuencias de la infección, como se observó en un estudio previo (Pinitkiatisakul et al., 2008). Por el contrario, esta asociación no fue observada tras la inmunización con el adyuvante Al/G. Aunque los mecanismos de acción de este adyuvante se desconocen, quizá la respuesta inmunitaria generada favoreció ciertas estrategias de evasión como la internalización del parásito en células migratorias del sistema inmunitario y la rápida diseminación a órganos inmunoprivilegiados como el cerebro, como se ha observado previamente en la infección por *T. gondii* (Lambert et al., 2006).

La dosis de antígeno utilizada en la inmunización es otro factor que puede influenciar el tipo de respuesta inmunitaria inducida y, por tanto, la eficacia inmunoprotectora de la vacuna. En nuestro estudio, la mayor protección se observó en los animales vacunados con la dosis intermedia. Concretamente, W/O combinado con 5×10^5 taquizoítos limitó la presencia del parásito en cerebro en la fase crónica y Al/G con 5×10^5 taquizoítos redujo la parasitemia en la fase aguda. Sin embargo, el efecto de la dosis administrada sobre la protección, únicamente se observó en los grupos inmunizados con Al/G, en los que durante la fase crónica la frecuencia y cargas parasitarias en el cerebro se incrementaron con la dosis. Un efecto similar por el empleo de altas dosis antigénicas fue observado por Baszler et al. (2000), quienes tras la inmunización de 50 µg de extracto solu-

ble de *N. caninum* detectaron una exacerbación de la infección cerebral. Por el contrario, los mejores resultados de protección se obtuvieron cuando se emplearon dosis más bajas (5 µg-25 µg, equivalentes a dosis de 10^5-5×10^5 taquizoítos) (Liddell et al., 1999; Ribeiro et al., 2009).

Los resultados obtenidos en el presente estudio muestran el efecto del adyuvante y la dosis antigénica sobre la eficacia de la inmunización. El adyuvante administrado juega un papel fundamental en el grado de protección, que parece ser más determinante que la dosis antigénica. A pesar de la gran dificultad para comparar los numerosos ensayos de inmunización con vacunas inactivadas frente a la neosporosis, debido al empleo de diferentes modelos experimentales que varían la estirpe murina utilizada, la dosis de inmunización, el adyuvante empleado, las características del desafío y los parámetros evaluados, en general, en la mayoría de estos estudios también destaca la influencia del adyuvante en la inducción de una respuesta protectora frente a la infección (Liddell et al., 1999; Lunden et al., 2002; Cannas et al., 2003b; Miller et al., 2005; Cardoso et al., 2011).

Para la consecución del siguiente subobjetivo (Subobjetivo 1.2; Capítulo III), las formulaciones que mejores niveles de protección mostraron en el modelo cerebral, se probaron en un modelo de infección congénita previamente estandarizado por el grupo SALUVET (López-Pérez et al., 2006; López-Pérez et al., 2008). Este modelo había mostrado ser adecuado para la evaluación de la eficacia de vacunas frente a la transmisión vertical (Aguado-Martínez et al., 2009; Marugán-Hernández et al., 2011b). Por un lado, se seleccionaron el adyuvante W/O y la dosis de 5×10^5 taquizoítos, ya que dicha combinación redujo la infección cerebral, sugiriendo una posible protección frente a la cronificación de la infección. Por otro lado, a pesar de los resultados obtenidos en la fase crónica, resultaba interesante probar el adyuvante Al/G y la dosis de 5×10^5 taquizoítos frente a la infección congénita, ya que sus efectos reductores de la parasitemia en la fase aguda podrían también ayudar a controlar la transmisión transplacentaria del parásito a la descendencia tras una reactivación o una primoinfección en la hembra gestante.

Adicionalmente, se evaluó el efecto de la inmunización con antígenos procedentes de diferentes estadios parasitarios en la eficacia frente a la infección congénita y cerebral. La hipótesis de partida fue que la inmunización con formulaciones que contuvieran una mezcla de antígenos procedentes de los estadios de taquizoíto y bradizoíto (TZ-BZ) podría facilitar que el sistema inmunitario reconociera proteínas implicadas en procesos claves en la patogenia de la enfermedad, como la conversión entre taquizoíto y bradizoíto, que conduce a la persistencia del parásito en el hospedador. Para ello, se compararon las formulaciones con taquizoítos inactivados, con otras elaboradas a partir de una mezcla de taquizoítos y bradizoítos obtenidos *in vitro*.

Para la obtención de los zoítos se seleccionó el aislado Nc-Spain 7, previamente obtenido en nuestro laboratorio (Regidor-Cerrillo et al., 2008), que en cultivo había mostrado una elevada cistogenicidad. La conversión de taquitoíto a bradizoíto se llevo a cabo mediante la adición al cultivo de nitroprusiato sódico, un agente químico estresante para el parásito (Risco-Castillo et al., 2004). Los zoítos se recogieron del cultivo en el día 7 tras la administración del agente estresante. Recientemente, un estudio proteómico reali-

zado por nuestro grupo de investigación utilizando el mismo protocolo de conversión de taquizoíto a bradizoíto, ha evidenciado la existencia de proteínas sobre-expresadas probablemente relacionadas con los sucesos implicados en el desarrollo temprano del estadio de bradizoíto (Marugán-Hernández et al., 2010).

Contrariamente a nuestro supuesto inicial, la inmunización con TZ-BZ indujo una exacerbación de la infección congénita y cerebral. Los elevados niveles de anticuerpos detectados frente a la proteína NcGRA7, implicada en procesos de replicación e invasión (Augustine et al., 1999; Cho et al., 2005), podrían estar relacionados con una elevada proliferación y diseminación del parásito, que explicaría la gravedad de los signos observados. También se detectó una elevada producción de anticuerpos frente a proteínas específicas del estadio de bradizoíto (NcSAG4 y NcBSR4), señalando una re-estimulación antigénica, probablemente debida al inicio del proceso de conversión de los parásitos tras el desafío. Desafortunadamente, en este estudio, no se emplearon técnicas que permitieran la detección de quistes tisulares, por tanto, si la vacunación con antígenos de TZ-BZ fue capaz de limitar el proceso de conversión y la cronificación de la infección en el cerebro se desconoce. Por otro lado, los animales vacunados con esta mezcla mostraron un incremento en la expresión de las citoquinas de tipo Th1 tras la inmunización, mientras que tras el desafío los animales mostraron un patrón completamente diferente, con una mayor expresión de citoquinas asociadas a una respuesta Th2. Una desviación excesiva hacia un tipo u otro de respuesta podría tener consecuencias negativas, agravando la enfermedad. Una sobreproducción de citoquinas proinflamatorias -Th1-, lejos de proteger de la infección por *N. caninum*, como han señalado numerosos autores (Khan et al., 1997; Yamane et al., 2000), podría estar asociada con una fuerte reacción inmunopatológica. Mientras, un exceso de respuesta tipo Th2 podría permitir una diseminación masiva del parásito. El mecanismo que condujo a la exacerbación no está claro, sin embargo, la rápida proliferación y diseminación del parásito en los animales inmunizados con TZ-BZ pudo ocasionar un desequilibrio en el balance Th1/Th2, generando una respuesta inmunitaria incapaz de controlar la infección.

Este trabajo destaca la influencia del tipo de antígeno en la protección conferida por una vacuna inactivada frente a la neosporosis. Hasta la fecha, este tipo de preparaciones han utilizado únicamente al taquizoíto como antígeno. Las escasas investigaciones realizadas hasta el momento que evalúan el papel de antígenos específicos de bradizoíto frente a la neosporosis congénita y cerebral han sido llevadas a cabo por el grupo SALUVET. Previamente, dos estudios evaluaron la eficacia protectora de la proteína específica del estadio bradizoíto (NcSAG4), por un lado, tras la inmunización con la proteína recombinante (Aguado-Martínez et al., 2009) y, por el otro, tras la vacunación con taquizoítos vivos de dos aislados genéticamente modificados de *N. caninum*, que expresaban constitutivamente esta proteína (Marugán-Hernández et al., 2011b). Los mejores resultados se observaron con la vacuna viva, aunque la inmunización con la proteína recombinante indujo un ligero retraso en la mortalidad neonatal. Considerando la complejidad de la respuesta inmunitaria generada frente a este apicomplejo, parece poco probable que la protección observada con la vacuna viva fuera consecuencia de la expresión de una sola proteína específica de estadio. No obstante, a pesar de que los resultados sobre el uso de

vacunas que contienen antígenos específicos de estadio obtenidos hasta la fecha no han mostrado buenos resultados, esta aproximación sigue siendo de interés. Posiblemente, el futuro de este tipo de vacunas pase por el desarrollo de nuevas formulaciones que contengan una mezcla de antígenos específicos de estadio o nuevos sistemas de adyuvantación.

Con respecto al papel de los adyuvantes, el presente trabajo está en concordancia con lo expuesto en el subobjetivo anterior y pone de manifiesto la importancia de estos compuestos en la eficacia de las vacunas inactivadas. La combinación de W/O y la dosis de 5×10^5 taquizoítos limitó parcialmente la transmisión vertical y la presencia del parásito en el cerebro, mientras que la combinación de Al/G y la dosis de 5×10^5 taquizoítos no fue capaz de reducir la transmisión vertical y la infección cerebral. De nuevo, la protección observada en el grupo de animales vacunados con W/O se relacionó con una elevada producción de anticuerpos, predominantemente de tipo Th2, y con una mayor expresión de IFN-γ, sugiriendo un equilibrio de la respuesta inmunitaria, favorable para el control de la infección.

El segundo objetivo de esta tesis doctoral fue el desarrollo de una vacuna viva frente a la infección por *N. caninum*, utilizando un aislado naturalmente atenuado. Las vacunas vivas han demostrado ser una de las herramientas más eficaces para la prevención y control de numerosas enfermedades producidas por protozoos apicomplejos en animales de renta (Meeusen et al., 2007). Una importante desventaja de estas vacunas son los riesgos potenciales que pueden derivarse de su empleo, relacionados con un mantenimiento de la virulencia residual o una reversión a ésta. Esta desventaja se ha tratado de solventar mediante la obtención de parásitos atenuados que presenten una limitada capacidad de persistencia en el organismo hospedador, disminuyendo el riesgo de acantonamiento, reactivación y transmisión de la infección. Esta estrategia ha sido utilizada con éxito en el desarrollo de vacunas frente a la toxoplasmosis ovina. De hecho, la única vacuna eficaz frente a esta enfermedad (Toxovax®, MSD Animal Health) utiliza un aislado avirulento (S48), atenuado mediante numerosos pases en cultivo celular, incapaz de desarrollar bradizoítos en el modelo murino e iniciar el ciclo sexual en el hospedador definitivo (familia Felidae). La alta eficacia de Toxovax®, probada tanto en estudios experimentales como en estudios de campo, se basa en que disminuye el número de abortos y la mortalidad neonatal en las ovejas vacunadas (Buxton et al., 1991; Buxton & Innes, 1995).

En la neosporosis, la aplicación de diversas metodologías ha permitido la obtención de aislados de virulencia reducida como el mutante termo-sensible Nc-1 ts-8 (Lindsay et al., 1999b), el aislado irradiado con rayos-γ Nc-1 528Gγ (Ramamoorthy et al., 2006), el aislado atenuado mediante pases en cultivo Nc-1 p.88 (Bartley et al., 2006) o los mutantes transgénicos Nc-1 SAG4^c 1.1 y 2.1 (Marugán-Hernández et al., 2011a). Todos ellos han sido probados como vacuna viva en diferentes modelos murinos, mostrando buenos resultados de protección (Lindsay et al., 1999b; Ramamoorthy et al., 2006; Ramamoorthy et al., 2007a; Ramamoorthy et al., 2007b; Bartley et al., 2008; Marugán-Hernández et al., 2011b). Sin embargo, se desconocen datos sobre su seguridad y eficacia en el ganado bovino, tanto en condiciones experimentales como en condiciones de campo. Curiosamente, la utilización del aislado Nc-Nowra de *N. caninum*, obtenido de forma natural mediante el aislamiento en cultivo celular del parásito a partir de un ternero asin-

tomático naturalmente infectado (Miller et al., 2002) ha mostrado los resultados más prometedores de protección frente a la infección congénita en un modelo murino (Miller et al., 2005) y frente al aborto tras la infección experimental en el ganado bovino (Williams et al., 2007). Si bien, como ocurre con el resto de aislados atenuados, los posibles efectos patógenos de este aislado cuando infecta al ganado bovino durante la gestación se desconocen.

Basado en la hipótesis de que un parásito obtenido a partir de un animal asintomático podría presentar cierta atenuación en su virulencia, nuestro primer objetivo pretendía responder a aquellos aspectos relacionados con la seguridad, de especial interés en este tipo de vacunas. Para ello, durante el desarrollo del Subobjetivo 2.1 (Capítulo IV), se llevó a cabo la obtención de un aislado de *N. caninum,* al que se denominó Nc-Spain 1H, a partir de un ternero clínicamente sano pero congénitamente infectado, como potencial candidato para la elaboración de una vacuna viva. Tras su obtención, y con el fin de garantizar la utilización de un preparado seguro, se realizaron pruebas de identificación genética y caracterización biológica, tanto *in vitro* como *in vivo,* que determinaron las propiedades biológicas intrínsecas del aislado.

En primer lugar, se realizó el estudio de 13 secuencias microsatélites previamente identificadas por nuestro grupo de investigación como marcadores de diversidad genética dentro de la especie *N. caninum* (Regidor-Cerrillo et al., 2006). Dicho estudio determinó un perfil genético para este aislado, diferente del obtenido para otros aislados analizados (Regidor-Cerrillo et al., 2006; Regidor-Cerrillo et al., 2008; Basso et al., 2009). La posibilidad de caracterizar genéticamente un aislado a partir de muestras biológicas (Pedraza-Díaz et al., 2009), podría ayudar a determinar la posible implicación del aislado Nc-Spain 1H en un problema de aborto o de transmisión vertical en un grupo de animales vacunados, ofreciendo una valiosa herramienta para garantizar la trazabilidad y la seguridad del aislado.

Posteriormente, durante la caracterización *in vitro*, el aislado de Nc-Spain 1H mostró un menor rendimiento en la producción de taquizoítos y una menor capacidad de infección en cultivos celulares en comparación con el aislado de referencia Nc-1. En concordancia con estos resultados, recientemente se ha descrito la reducida capacidad de invasión y una baja tasa de proliferación de este aislado (Regidor-Cerrillo et al., 2010). Asimismo, el aislado Nc-Spain 1H mostró una tasa de conversión taquizoíto-bradizoíto similar a la observada en el aislado Nc-Liv, con la diferencia de que el aislado Nc-Spain 1H únicamente produjo bradizoítos intermedios. La obtención del aislado Nc-Spain 1H a partir del cerebro de un ternero congénitamente infectado indica su capacidad de conversión de taquizoíto a bradizoíto. Sin embargo, la ausencia de vacuolas conteniendo bradizoítos puros *in vitro* podría indicar una menor capacidad de persistencia de este aislado, ya que este proceso de conversión parece estar relacionado con la capacidad de evasión del sistema inmunitario y cronificación de la infección en el hospedador, tal y como se ha observado en *T. gondii* (Kim & Boothroyd, 2005; Kim et al., 2007; Saeij et al., 2008).

Los resultados de los ensayos *in vitro* están en consonancia con los resultados obtenidos *in vivo* en sendos modelos murinos de neosporosis cerebral (Collantes-Fernández

et al., 2006b) y congénita (López-Pérez et al., 2006; López-Pérez et al., 2008). Por un lado, el aislado Nc-Spain 1H, inoculado a diferentes dosis, no indujo signos clínicos ni mortalidad y no se detectó en el cerebro durante la fase crónica de la infección, incluso utilizando altas dosis del parásito (10^7 taquizoítos). Por otro lado, tras su inoculación durante el segundo tercio de la gestación, el aislado Nc-Spain 1H indujo una baja mortalidad neonatal y una reducida transmisión vertical en comparación con el aislado de referencia Nc-1. La relación entre una reducción en la capacidad invasiva y en la tasa de replicación y una menor virulencia en un modelo de infección en el modelo murino gestante ha sido descrita recientemente en varios aislados obtenidos de forma natural a partir de animales asintomáticos, entre ellos el aislado en estudio (Regidor-Cerrillo et al., 2010). Esta menor capacidad de invasión y proliferación observada *in vitro* podría sugerir una menor habilidad de estos aislados para cruzar barreras biológicas (hemato-encefálica o placentaria) y para invadir y multiplicarse en diferentes tejidos, reduciendo el daño tisular y los signos clínicos asociados a la infección, explicando la baja patogenicidad del aislado Nc-Spain 1H observada *in vivo*.

Los caracterización de un aislado en un modelo murino es el primer abordaje para seleccionar potenciales candidatos para la elaboración de una vacuna (Collantes-Fernández et al., 2004; López-Pérez et al., 2006; López-Pérez et al., 2008; Pereira García-Melo et al., 2010; Regidor-Cerrillo et al., 2010; Marugán-Hernández et al., 2011a). No obstante, previamente a los ensayos de inmunización, los posibles candidatos deberían ser inoculados en la especie de destino con el fin de comprobar que dichos aislados conservan las características de atenuación observadas en los modelos de laboratorio. Con este propósito, en el Subobjetivo 2.2 (Capítulo V), se llevó a cabo la caracterización patogénica del aislado Nc-Spain 1H en un modelo bovino gestante.

La capacidad del aislado Nc-Spain 1H para transmitirse verticalmente y producir mortalidad fetal, se evaluó en un modelo bovino gestante de infección experimental previamente validado para los aislados Nc-Liv y Nc-1 en el que la inoculación de estos aislados por vía intravenosa en vacas en el día 70 de gestación causó la muerte del feto (Williams et al., 2000; Macaldowie et al., 2004). Este modelo se eligió por su elevada eficacia para reproducir la mortalidad fetal asociada a la infección por *N. caninum,* resultando ser una herramienta útil para el estudio del comportamiento del aislado Nc-Spain 1H en el ganado bovino mediante su comparación con el aislado de referencia Nc-1.

En relación a la patogenicidad, la infección con el aislado Nc-Spain 1H en el día 70 de gestación no produjo mortalidad fetal. Tampoco se detectó al parásito en los tejidos fetales y las lesiones observadas en placenta y órganos fetales fueron más leves que las encontradas en los animales infectados con el aislado Nc-1. En nuestro estudio, las lesiones encontradas en los animales que presentaron mortalidad fetal tras la inoculación con Nc-1 concuerdan con las descritas en otras infecciones experimentales realizadas en el primer tercio de gestación utilizando los aislados Nc-1 y Nc-Liverpool (Williams et al., 2000; Innes et al., 2001; Macaldowie et al., 2004; Maley et al., 2006; Gibney et al., 2008). En dichos estudios, en aquellos animales en los que ocurrió la muerte fetal, se describieron graves lesiones en tejidos placentarios y fetales asociadas a la presencia del parásito, sugiriendo una diseminación incontrolada de éste en el feto, cuyo sistema inmu-

nitario es todavía inmaduro. En estudios más recientes, donde las infecciones se realizaron en los días 70 y 110 de gestación, las graves lesiones encontradas en la placenta de los animales que presentaron muerte fetal se asociaron a una amplia distribución del parásito y a un dramático incremento en la expresión y producción de citoquinas de tipo Th1 y Th2 en la placenta y en el feto (Rosbottom et al., 2008; Almeria et al., 2012). Sin embargo, dicha respuesta inmunitaria no se observó cuando el feto sobrevive. Se desconoce si la muerte fetal se debe principalmente al daño tisular directo del parásito o si también interviene el efecto citopático de las citoquinas producidas en placenta, probablemente para limitar la diseminación del parásito al feto. El comportamiento *in vitro* del aislado Nc-Spain 1H podría explicar una menor capacidad de multiplicación intracelular *in vivo,* que por un lado limitaría el daño causado por la replicación del parásito en los tejidos placentarios y fetales y, por otro, generaría un menor estímulo antigénico que reduciría la respuesta inmunitaria en la interfase materno-fetal, evitando su posible efecto negativo sobre la gestación. Esto explicaría las diferencias encontradas entre las repercusiones causadas por el aislado en estudio y el aislado de referencia Nc-1.

Por otro lado, los niveles de anticuerpos y de IFN-γ producidos específicamente tras la infección con taquizoítos del aislado Nc-Spain 1H fueron menores que los observados en las novillas infectadas con el aislado Nc-1. La menor magnitud de esta respuesta en comparación con la inducida por el Nc-1 y con lo descrito por otros autores tras la infección con los aislados Nc-1 o Nc-Liverpool (Williams et al., 2003; Bartley et al., 2004), nuevamente pone de manifiesto un menor estímulo antigénico por parte del aislado en estudio. Estos resultados concuerdan con los obtenidos previamente durante la caracterización en ratona gestante, donde los niveles de anticuerpos inducidos por el aislado Nc-Spain 1H fueron significativamente más bajos que los del grupo infectado con el aislado Nc-1.

Los resultados anteriores indican una baja virulencia del aislado Nc-Spain 1H tanto en un modelo de laboratorio como en la especie de destino, presentándose como un candidato seguro para el desarrollo de una vacuna viva. Con todos estos antecedentes, en el Subobjetivo 2.3 (Capítulo VI), se llevó a cabo un ensayo para valorar la seguridad y la eficacia de dicho aislado frente a la neosporosis congénita y cerebral en un modelo murino. Complementariamente, se realizó la evaluación de la eficacia de diferentes dosis del parásito vivo con la finalidad de conocer si el grado de protección y el tipo de respuesta inmunitaria de la vacuna eran dependientes de la dosis.

En el presente estudio, la inmunización de ratones con 5×10^5 taquizoítos del aislado Nc-Spain 1H indujo una respuesta inmunitaria protectora capaz de controlar la transmisión vertical a la descendencia y la infección cerebral tras el desafío durante la gestación con el aislado heterólogo Nc-Liverpool. Esta elevada protección frente a la infección, por encima del 90%, se asoció a una producción de IFN-γ, aunque también se observaron la producción de citoquinas relacionadas con una respuesta de tipo Th2 (IL4 e IL10) y el desarrollo de elevados niveles de anticuerpos. En estudios previos se han reportado niveles similares de protección frente a la transmisión congénita tras la inmunización con taquizoítos vivos de los aislados Nc-Nowra (Miller et al., 2005) y NcSAG4^c 2.1 (Marugán-Hernández et al., 2011b). De la misma manera, numerosos ensayos de vacuna-

ción con parásitos vivos realizados en modelos de infección cerebral han mostrado altos niveles de protección (Lunden et al., 2002; Ramamoorthy et al., 2006; Ramamoorthy et al., 2007a). La protección observada en todos estos estudios estuvo asociada a una respuesta inmunitaria sin una clara predominancia de un patrón Th1 o Th2. Aunque resulta difícil comparar la eficacia inmunoprotectora entre los distintos ensayos vacunales debido a la gran variedad de modelos experimentales empleados, la protección observada tanto en estos estudios como la conferida por el aislado Nc-Spain 1H, parece relacionarse con un equilibrio entre ambos tipos de respuesta, Th1 y Th2. La inducción de una respuesta inmunitaria equilibrada podría ser determinante para limitar la multiplicación del parásito, protegiendo al hospedador sin originarle el daño tisular resultante de una respuesta inflamatoria exacerbada.

Tras confirmar la eficacia protectora del aislado Nc-Spain 1H frente a las infecciones congénita y cerebral en ratón, se evaluó el efecto de la dosis de inmunización sobre la protección frente a la transmisión congénita. Para ello, se probaron cinco dosis de taquizoítos del aislado Nc-Spain 1H, desde 5×10^5 hasta 5×10^1. Todas las dosis inoculadas confirieron una protección elevada, por encima del 60%. Sorprendentemente, el grupo inoculado con la dosis más baja mostró la mayor protección. Tras el análisis estadístico, no se observaron diferencias entre los grupos vacunados, indicando que la protección conferida por el aislado no fue dependiente de la dosis de inmunización. Estos resultados son de sumo interés para el desarrollo de una vacuna viva, en términos de seguridad y de producción a nivel industrial, ya que ofrecen la posibilidad de reducir la dosis administrada de taquizoítos vivos. Esto cobra especial importancia tratándose de una vacuna cuya especie de destino es el ganado bovino, donde la dosis a utilizar aumentará en varios logaritmos con respecto a la utilizada en los ensayos en el modelo murino (Miller et al., 2005; Williams et al., 2007).

Sin embargo, sí se observó una influencia de la dosis de inmunización sobre la respuesta inmunitaria inducida. La dosis más baja del parásito dio lugar a una producción predominante de anticuerpos del subisotipo IgG2a, mientras que las dosis más altas se asociaron a elevados niveles de IgG1. A pesar de los diferentes perfiles de anticuerpos observados, todas las dosis probadas del aislado Nc-Spain 1H indujeron una elevada protección. De manera similar, Lunden et al. (2002) describen que la inmunización con dos dosis subletales diferentes del aislado Nc-1 confiere protección, a pesar de inducir patrones de anticuerpos diferentes. Aunque los mecanismos por los que el parásito modula la respuesta inmunitaria no están claros, en ambos casos la dosis altas del parásito indujeron una protección asociada a una respuesta mixta Th1-Th2, que combinó una producción predominante de IFN-γ y una respuesta de anticuerpos mayoritariamente de tipo IgG1. Dicho equilibrio pudo ser beneficioso, como se señaló anteriormente, para el control tanto de la multiplicación del parásito como del daño producido por la respuesta generada. En cambio, la inoculación de dosis bajas del parásito podría favorecer la internalización de la mayoría de los parásitos en las células presentadoras de antígeno, quedando pocos taquizoítos extracelulares, favoreciendo una respuesta de base celular, con una producción predominante de IgG2a.

Como se ha expuesto anteriormente, una de las mayores desventajas de la utilización de una vacuna viva son los problemas de seguridad derivados del posible acantonamiento, reactivación y transmisión del aislado con que se inmuniza. Con respecto a la seguridad del aislado Nc-Spain 1H, en este trabajo no se detectó la presencia de ADN del parásito en el cerebro de ninguno de los animales inmunizados. Estos datos están de acuerdo con lo observado en los estudios previos de caracterización del aislado en sendos modelos murino y bovino, y parecen confirmar la baja virulencia y persistencia de este aislado en el hospedador. Recientemente, Marugán-Hernández et al. (2011b) han señalado una correspondencia entre la baja virulencia y la reducida capacidad de invasión de un aislado obtenido mediante genética reversa (NcSAG4^{c} 1.1) y su baja capacidad inmunoprotectora, sugiriendo que un retraso en la invasión y la menor diseminación en el organismo de este aislado podrían estar induciendo una respuesta inmunitaria protectora de menor eficacia. A diferencia de ese trabajo, los presentes experimentos muestran que el aislado Nc-Spain 1H es un aislado seguro, como muestra la completa eliminación del cerebro y, al mismo tiempo, capaz de inducir una respuesta inmunitaria que confiere altos niveles de protección frente a la infección.

En resumen, teniendo en cuenta todos los resultados obtenidos en la presente tesis doctoral, la inmunización con taquizoítos vivos del aislado Nc-Spain 1H parece conferir una mayor protección que las formulaciones a partir de parásito inactivado, mostrándose como una estrategia prometedora para el desarrollo de una vacuna segura y eficaz frente a la neosporosis en el ganado bovino. Hasta la fecha se han realizado pocos estudios de inmunización en esta especie. De hecho, en la actualidad no existe ninguna vacuna en el mercado y, sólo una vacuna inactivada (NeoGuard®, Intervet), estuvo aprobada para su uso en vacas gestantes en los Estados Unidos hasta el año 2009. A pesar de ser una vacuna segura, su papel protector frente al aborto fue muy discutido, ya que no confería protección en vacas gestantes infectadas experimentalmente con el parásito (Andrianarivo et al., 2000), y en varios estudios de campo presentó una eficacia variable (5,2%-54%) (Heuer et al., 2003; Romero et al., 2004; Weston et al., 2011). Experimentalmente, se han observado excelentes resultados de protección tras la inmunización con el aislado Nc-Nowra. Los resultados obtenidos demuestran una reducción drástica de la transmisión vertical tras el desafío heterólogo en un modelo murino gestante (Miller et al., 2005). En el modelo bovino gestante, dio lugar a una protección total frente a la muerte fetal tras el desafío en el día 70 de gestación con el aislado Nc-Liverpool (Williams et al., 2007). Sin embargo, se desconocen datos sobre la seguridad del aislado en animales gestantes. Por tanto, los resultados obtenidos en esta tesis doctoral abren la perspectiva de realización de nuevos estudios que determinen la seguridad y la eficacia protectora del aislado Nc-Spain 1H frente al aborto y la transmisión vertical en un modelo bovino gestante, tanto en condiciones experimentales como en pruebas de campo.

Capítulo VIII

Objetivo 1. Desarrollo de vacunas inactivadas frente a la neosporosis basadas en la utilización de zoítos de *N. caninum*.

Primera. La inmunización de ratones con zoítos inactivados de *N. caninum* induce una protección parcial frente la neosporosis congénita y cerebral. El grado de protección conferido por estas formulaciones inactivadas viene determinado, fundamentalmente, por dos factores, el adyuvante y el tipo de antígeno.

Segunda. La emulsión de agua en aceite combinada con taquizoítos enteros inactivados ha mostrado los mejores resultados de protección entre todas las formulaciones inactivadas ensayadas, controlando parcialmente la infección cerebral y congénita. Por el contrario, la inmunización con hidróxido de aluminio combinado con CpG o extracto de Ginseng induce una respuesta inmunitaria incapaz de proteger frente a la infección por *N. caninum*.

Tercera. La vacunación con una mezcla de taquizoítos y bradizoítos inactivados induce una exacerbación de la infección cerebral y congénita, asociada a una potente respuesta inmunitaria definida por un desequilibrio en la expresión de citoquinas que podría ser perjudicial para el hospedador.

Objetivo 2. Desarrollo de una vacuna viva frente a la neosporosis basada en la utilización de un aislado naturalmente atenuado de *N. caninum*

Primera. La obtención y caracterización de aislados de *N. caninum* a partir de terneros congénitamente infectados pero clínicamente sanos ha resultado ser una estrategia acertada para la identificación de aislados candidatos para el desarrollo de una vacuna viva atenuada frente a la neosporosis bovina. Se ha obtenido un nuevo aislado denominado Nc-Spain 1H, que presenta un perfil genético definido y exclusivo.

Segunda. El aislado Nc-Spain 1H de *N. caninum* muestra una menor persistencia en el modelo murino cerebral y una reducida capacidad de transmisión transplacentaria en los modelos murino y bovino gestantes. La menor patogenicidad observada *in vivo* está relacionada con una baja capacidad de invasión y proliferación *in vitro*. Esta reducida virulencia señala al aislado Nc-Spain 1H como un candidato seguro para el desarrollo de una vacuna viva atenuada frente a la neosporosis bovina.

Tercera. La inmunización con taquizoítos vivos del aislado Nc-Spain 1H resulta segura y estimula una respuesta inmunitaria humoral y celular capaz de bloquear eficazmente la multiplicación del parásito y su transmisión vertical en un modelo murino. La elevada protección conferida por dicho aislado es independiente de la dosis de vacunación utilizada, aunque parece que este factor influencia los mecanismos inmunitarios que conducen a tal protección.

Objective 1. Development of inactivated whole vaccines against neosporosis

Conclusion 1. The immunization of mice with inactivated whole *N. caninum* zoites induces partial protection against congenital and cerebral neosporosis. The protection conferred by these inactivated vaccines is mainly influenced by two factors: the adjuvant and the type of antigen.

Conclusion 2. The water-in-oil emulsion combined with inactivated whole tachyzoites induces higher protection than any of the other evaluated inactivated formulations, partially preventing congenital and cerebral neosporosis. Conversely, the immune response elicited by immunization with aluminum hydroxide plus CpG-ODN or Ginseng extract does not protect against *N. caninum* infection.

Conclusion 3. The immunization with a mixture of inactivated whole tachyzoites and bradyzoites causes an exacerbation of congenital and cerebral neosporosis, which is related to a strong immune response characterized by an imbalanced cytokine expression that could be harmful to the host.

Objective 2. Development of live vaccines against neosporosis by using a naturally attenuated isolate of *N. caninum*

Conclusion 1. The isolation and characterization of *N. caninum* isolates obtained from congenitally infected but clinically healthy calves has been proven a convenient approach to identify candidates for the development of a live vaccine against bovine neosporosis. A new isolate of *N. caninum* has been obtained: Nc-Spain 1H, whose genetic profile is defined and exclusive.

Conclusion 2. The Nc-Spain 1H isolate of *N. caninum* shows low persistence in mice brains and a reduced transplacental transmission in both pregnant mouse and bovine models. Its moderate *in vivo* pathogenicity can be related to the low invasion and proliferation rates observed *in vitro*. This low virulence points to the Nc-Spain 1H isolate as a safe candidate for the development of an attenuated live vaccine against bovine neosporosis.

Conclusion 3. Immunization with live Nc-Spain 1H tachyzoites seems to be safe and induces humoral and cellular immune responses that can block efficiently the multiplication of the parasite and its vertical transmission in mouse models. The high protection conferred by the isolate does not depend on the inoculated dose of live tachyzoites, although this factor seems to influence the immune mechanisms leading to such protection.

Resumen/Summary

Neospora caninum es un parásito intracelular formador de quistes considerado mundialmente como una de las principales causas de aborto en el ganado bovino. La magnitud de las pérdidas económicas asociadas a la neosporosis bovina, junto con la ausencia de medidas económicamente viables para su control, han conducido a que uno de los principales retos en la investigación sobre esta enfermedad sea el desarrollo de una vacuna eficaz, segura y rentable frente al aborto y la transmisión del parásito.

En los últimos años, se han llevado a cabo numerosos estudios para el desarrollo de vacunas frente a las neosporosis bovina, incluyendo vacunas inactivadas, vivas, y de nueva generación basadas en proteínas recombinantes, en vectores vivos o en vacunas de ADN. Las vacunas de nueva generación ofrecen alternativas interesantes para el futuro pero los resultados de protección obtenidos hasta el momento son insuficientes. Por el contrario, las únicas vacunas que hasta la fecha se han probado en la especie de destino con cierto éxito utilizan parásitos inactivados y vivos atenuados, hecho que dio pie a la realización de la presente tesis doctoral.

En la primera parte del estudio, se evaluó el efecto protector de formulaciones empleando parásito entero inactivado como antígeno. En este tipo de vacunas, la selección apropiada de determinadas variables, como el tipo de antígeno o el adyuvante, puede ser determinante para la inducción de una respuesta inmunoprotectora frente a la infección por *N. caninum*. Por ello, en un primer experimento se evaluó la influencia de tres adyuvantes (emulsión oleosa de agua en aceite -W/O- e hidróxido de aluminio combinado con CpG -Al/CpG- o extracto de Ginseng -Al/G-) en combinación con tres dosis diferentes de taquizoítos enteros inactivados (10^5, 5×10^5 y 10^6) en un modelo murino de neosporosis cerebral (Capítulo II). En este estudio se puso de manifiesto la influencia del adyuvante en la eficacia protectora de la vacunación. Así, la inmunización de W/O combinado con 5×10^5 taquizoítos limitó la presencia del parásito en el cerebro en la fase crónica y, por tanto, la posible cronificación y persistencia de la infección en este órgano, mientras que el adyuvante Al/G combinado con 5×10^5 taquizoítos redujo la parasitemia durante la fase aguda. La dosis de antígeno afectó, aunque de forma menos notable que el adyuvante, la eficacia protectora de la vacunación, de manera que en los grupos inmunizados con Al/G, la frecuencia y cargas parasitarias en el cerebro se incrementaron con la dosis.

Para el siguiente estudio (Capítulo III), se seleccionaron los adyuvantes y dosis de antígeno que mejores niveles de protección mostraron en el modelo cerebral, y se estudió su capacidad protectora en un modelo de infección congénita. Por un lado, se seleccionaron el adyuvante W/O y la dosis de 5×10^5 taquizoítos, ya que dicha combinación redujo la infección cerebral, sugiriendo una posible protección frente a la cronificación de la infección. Por otro lado, se probó el adyuvante Al/G y la dosis de 5×10^5 taquizoítos frente a la infección congénita, ya que sus efectos reductores de la parasitemia en la fase aguda podrían también ayudar a controlar la transmisión transplacentaria del parásito a la descendencia tras una reactivación o una primoinfección en la hembra gestante. Adicionalmente, se comparó el efecto entre estas formulaciones y aquellas elaboradas a partir de una mezcla de antígenos procedentes de los estadios de taquizoíto y bradizoíto (TZ-BZ). La hipótesis de partida fue que estas últimas pudieran conferir protección frente a los

procesos de reactivación en los que está implicado el estadio de bradizoíto. Sin embargo, la inmunización con la mezcla de TZ-BZ mostró los peores resultados de protección, originando una exacerbación de las infecciones cerebral y congénita, asociada a un probable desequilibrio de la respuesta inmunitaria caracterizado por una sobreexpresión de citoquinas de tipo Th1 tras la inmunización y de tipo Th2 tras el desafío. Por el contrario, la combinación de W/O y taquizoítos indujo un equilibrio de la respuesta inmunitaria caracterizado por una elevada producción de anticuerpos, predominantemente de tipo Th2, y con una sobreexpresión de IFN-γ, que controló parcialmente la infección cerebral y la transmisión vertical.

En la segunda parte del estudio, se investigó el desarrollo de una vacuna viva atenuada frente a la infección por *N. caninum*. Para ello, la estrategia de partida fue la obtención y caracterización de un aislado a partir de un animal asintomático que mostrara cierta atenuación en su virulencia (Capítulos IV y V). Como fruto de esta aproximación, se obtuvo el aislado Nc-Spain 1H de *N. caninum* a partir de un ternero clínicamente sano pero congénitamente infectado. Dicho aislado se identificó genéticamente mediante el análisis de 13 secuencias microsatélites previamente identificadas en el genoma de *N. caninum,* mostrando un patrón definido y exclusivo.

Los ensayos de caracterización *in vitro* mostraron un bajo rendimiento en la producción de taquizoítos y una menor capacidad de infección del aislado Nc-Spain 1H en comparación con el aislado de referencia Nc-1. Durante su caracterización biológica *in vivo*, el aislado Nc-Spain 1H mostró una virulencia notablemente reducida en un modelo murino de infección cerebral y congénita, caracterizada por una menor persistencia en el cerebro de ratones y una virulencia reducida con ausencia de signos clínicos y bajas tasas de mortalidad y transmisión vertical de la infección a las crías. En un modelo bovino gestante, dicho aislado no produjo mortalidad fetal y no se detectó presencia del parásito en los tejidos fetales.

Los resultados previamente obtenidos, en su conjunto, señalaron al aislado Nc-Spain 1H como un buen candidato para el desarrollo de una vacuna viva atenuada puesto que demostró ser seguro tanto en un modelo de laboratorio como en la especie de destino. El siguiente paso consistió en valorar la seguridad y la eficacia de dicho aislado frente a la neosporosis congénita y cerebral en un modelo murino inducidas mediante desafío con un aislado heterólogo virulento (Capitulo VI). Complementariamente, se evaluó la influencia de la dosis de inmunización (número de parásitos vivos administrados) en la eficacia de la vacunación en este modelo experimental. En el presente estudio, la inmunización de ratones con una dosis elevada de taquizoítos vivos del aislado Nc-Spain 1H confirió elevada protección, superior al 90%, frente a la transmisión vertical a la descendencia y la infección cerebral. Dicha protección se asoció a una producción de citoquinas, tanto de tipo Th1 como Th2, en la que predominaba el IFN-γ. Este resultado indicaba un equilibrio entre ambos tipos de respuesta capaces de limitar la multiplicación del parásito sin ser perjudiciales para el hospedador. Al evaluar el efecto de la dosis de inmunización sobre la eficacia de la vacunación, no se observó influencia de este factor, aunque la dosis inoculada pareció modular el tipo de respuesta inmunitaria inducida.

Los resultados obtenidos durante la ejecución de esta tesis doctoral han permitido la identificación y selección de candidatos para el desarrollo de vacunas inactivadas y vivas atenuadas frente a la neosporosis bovina, aportando a la comunidad científica nuevos datos sobre los factores que determinan la eficacia protectora de dichas vacunas. El hallazgo más destacable es el desarrollo de una vacuna viva atenuada basada en la inmunización con taquizoítos vivos del aislado naturalmente atenuado Nc-Spain 1H. Este hecho abre la puerta a nuevos estudios de seguridad y de eficacia protectora frente al aborto y la transmisión vertical de *N. caninum* en el ganado bovino y supone una prometedora herramienta para el control de la enfermedad en esta especie doméstica.

Neospora caninum is an intracellular cyst-forming parasite known as a major cause of bovine abortion worldwide. Due to the negative economic impact of bovine neosporosis, together with the lack of cost-effective measures for its control, the development of an economically viable, efficacious and safe vaccine against abortion and parasite transmission has become one of the main challenges for the research of this disease.

In the last few years, many studies have been carried out with the aim of developing vaccines against bovine neosporosis, including live, inactivated, and new generation protein recombinant, live vector or DNA vaccines. Up to now, new generation vaccines offer an interesting potential for the future, but have shown insufficient protection results. Contrarily, inactivated and live attenuated vaccines are the only ones that have been tested in bovine models with some success so far. This fact motivated the present Doctoral Thesis.

The first part of the study evaluated the protection conferred by vaccines containing inactivated whole parasites as antigen. In these types of formulations, an appropriate selection of certain variables, such as the type of antigen or the adjuvant, may determine the induction of a protective immune response against *N. caninum* infection. Therefore, the first experiment examined the role of three different adjuvants (water-in-oil emulsion -W/O- and aluminum hydroxide plus CpG-ODN -Al/CpG- or plus ginseng extract -Al/G) combined with three different doses of whole inactivated tachyzoites (10^5, 5×10^5 and 10^6) using a mouse model of cerebral neosporosis (Chapter I). The results highlighted the influence of the adjuvant on the protective efficacy of the vaccine. Thus, immunization with W/O combined with 5×10^5 tachyzoites limited parasite presence in the brain during the chronic stage of the infection and, consequently, the possibility that the infection becomes chronic and persistent in this organ. Contrarily, Al/G plus 5×10^5 tachyzoites reduced parasitaemia during the acute stage of the infection. The antigen dose played a role in the protective efficacy of the vaccine, although it was less significant than the adjuvant's: among the groups immunized with Al/G, parasite frequency and load in the brain rised as the dose was increased.

These results suggest that the W/O may control the establishment of infection in the brain, whereas the Al/G may control the vertical transmission of the parasite to progeny by reducing maternal parasitaemia and therefore the number of parasites reaching the placenta and fetus. For that reason, the co-administration of the W/O and Al/G adjuvants with the 5×10^5 tachyzoite dose was selected for the next experiment (Chapter II), in which its protective role in congenital infection models was evaluated. Additionally, the effect of these formulations was compared to that of vaccines based on a mixture of antigens from the tachyzoite and bradyzoite stages (TZ-BZ). The preliminary hypothesis was that the latter could confer protection against the reactivation processes in which the bradyzoite stage takes part. Nevertheless, immunization with a TZ-BZ mixture lead to the worst protection results, exacerbating congenital and cerebral neosporosis probably as a consequence of the induction of an imbalanced immune response due to a Th1-type cytokine overexpression after immunization and a Th2-type one after challenge. Contrarily, the immunization with W/O and tachyzoites induced a balanced immune response charac-

terized by a high antibody production, mainly type Th2, and by an IFN-γ overexpression, which partially controlled cerebral infection and vertical transmission.

The second part of the study addressed the development of a live attenuated vaccine against *N. caninum* infection. In order to achieve that, the initial strategy focused on obtaining and characterizing an isolate from an asymptomatic animal that stood out because of an attenuated virulence (Chapters III and IV). As a result of this approach, a new isolate of *N. caninum*, named Nc-Spain 1H, was obtained from the brain of a clinically healthy but congenitally infected calf. Such isolate, which was genetically identified through the analysis of 13 microsatellite sequences that had been previously identified in the *N. caninum* genome, showed a defined and exclusive profile.

The *in vitro* characterization of Nc-Spain 1H revealed a lower tachyzoite yield and viability rate than those of the reference Nc-1 isolate. During its *in vivo* biological characterization, the Nc-Spain 1H isolate showed a significantly reduced virulence in mouse models of cerebral and congenital neosporosis: it was less persistent in mouse brains and displayed a reduced virulence with no clinical signs and low neonatal mortality and vertical transmission rates. In a pregnant bovine model, Nc-Spain 1H did not cause fetal death and no evidence of parasite presence in fetal tissues was found.

On the whole, these results pointed to the suitability of Nc-Spain 1H as a candidate for the development of a live attenuated vaccine, since it has been proven safe in both mouse and bovine models. The following step was the evaluation of the safety and efficacy of the isolate against congenital and cerebral neosporosis induced in a mouse model through challenge with a virulent heterologous isolate (Chapter V). In addition, the influence of different immunising doses (number of inoculated live parasites) on the vaccine's efficacy was established. In the present study, immunization with a high dose of live Nc-Spain 1H tachyzoites conferred an elevated protection (higher than 90%) against vertical transmission and cerebral neosporosis in the models. This protection was associated with both Th1- and Th2-type cytokine production in which IFN-γ predominated. This result indicated a balance between the two types of response that was capable of limiting parasite multiplication without harming the host. The immunising dose was found to have no influence on the vaccine's efficacy, although the inoculated dose seemed to modulate the type of induced immune response.

The results of this Doctoral Thesis have allowed us to identify and select candidates for the development of inactivated and live attenuated vaccines against bovine neosporosis, thus providing the scientific community with new data about the factors determining the protective efficacy of such vaccines. The most remarkable finding is the development of a live attenuated vaccine based on immunization with live tachyzoites of the naturally attenuated Nc-Spain 1H isolate of *N. caninum*. This finding opens the door to further studies on safety and protective efficacy against *N. caninum*-associated abortion and vertical transmission of this parasite in cattle and, therefore, it becomes a promising tool for the control of this bovine disease.

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